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of the  
Conference on  
Nursery Production of Fruit Plants  
Through Tissue Culture -  
Applications and Feasibility

April 21-22, 1980  
Beltsville, Maryland

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PROCEEDINGS  
OF THE  
CONFERENCE ON  
NURSERY PRODUCTION OF FRUIT PLANTS  
THROUGH TISSUE CULTURE -  
APPLICATIONS AND FEASIBILITY

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# Mass Propagation by Tissue Culture:

## Principles and Techniques

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During the last 6 years many review articles have been published on tissue culture propagation of plants. Dr. Murashige has been the major contributor and has thoroughly established principles and concepts of the tissue culture method. Murashige (7) developed the concepts of developmental stages. These now include Stage 1 - explanting, or the establishment of live and growing plant material in culture; Stage 2 - multiplication of the propagule in culture; Stage 3 - establishment of rooted plants and hardening them off to survive planting into soil; and Stage 4 - the planting into soil and special treatment required for initiating rapid growth and development. He also discussed the physical and chemical requirements of the culture media and culture room environment for the tissue culture stages.

Murashige (9, 10) detailed the 3 types of propagule multiplication that are possible through tissue culture: (1) enhanced axillary bud breaking, (2) production of adventitious shoots, and (3) somatic cell embryogenesis. Enhanced axillary bud breaking perhaps nets the lowest multiplication rates as the maximum number of shoots produced is limited to the number of axillary buds planted in each culture. Adventive shoots have a greater potential in multiplication as shoots may arise from any area of the recultured inoculum. Somatic cell embryogenesis has potentially the greatest multiplication rates and produces a complete tiny plant. At present there are many basic and technical problems that must be resolved before this system will be commercially feasible. Tisserat et al. (14) reviewed the subject of somatic embryogenesis.

The production of disease-free plants through tissue culture and heat therapy has been reviewed by Murashige (8) and more recently by Stone (13).

References of literature on tissue culture of specific crops are readily obtained in Murashige's 1974 and 1978 reviews.

The emphasis of my discussion will be focused on 3 problem areas that appear to be impeding the commercial tissue culture development in fruit crops. These are (1) difficulty in successfully establishing explants in culture, (2) refinements of culture methodology for consistent high plant yields, and (3) induction of root initiation and acclimatization of the propagules in the soil stage. The explanting and rooting problems are of major importance to the woody fruit plants, while refinement of culture methodology is of utmost importance for all commercial propagation

endeavors. Boxus (3) correctly stated that obtaining high yields is "related to a range of apparently insignificant factors, which are in fact extremely important".

## STAGE 1: Explanting

The choice of explant may be a critical factor in establishing stock cultures. The shoot apex, 0.1-1.0 mm long, has been the most frequently used explant material. The obvious advantage of using small apices as explants is the probability of eliminating many pathogens from the cultures. Sometimes survival of small shoot apices are totally unsuccessful and other explant sources must be considered.

Long, softwood shoot tips, 2-5 cm long, are sometimes a viable alternative. This is particularly true when small shoot apex cultures rapidly turn brown and stain the media. Larger shoot tips reduce the ratio of wounded surface area to total surface area involved. Another advantage of large shoot tips is the apparent availability of stored food reserves and growth regulators in the stem that is helpful in the initiation of new growth.

Another potential source of explants is etiolated shoots arising from root cuttings. Some plants, such as red raspberry, readily sucker from the roots. Raspberry root cuttings can be placed in a humid environment in darkness and the root nodules will sprout shoots from which the shoot tips and axillary buds are easily explanted with a high rate of success.

Small seedlings may be used as a final source of explant material when other avenues have been exhausted. Frequently seedlings can be successfully explanted and used to determine the appropriate medium and environmental conditions for propagation, rooting and establishment in soil. Once a proven system is established then probability of successful explanting of clonal material is increased.

Another area of concern is the attainment of cultures free of algae, bacteria, fungi and other microorganisms. The normal surface sterilization with diluted laundry bleach of about 0.5-1% sodium hypochlorite with the addition of a few drops of surfactant per 100 ml of diluted solution is an effective surface disinfestation of plant material. The reaction is basically a dosage response of time and concentration. The use of ethanol as a short-term pre-soak is sometimes helpful with plant materials that are difficult to get adequate wetting of the surface. Mechanical agitation or the use of a gentle vacuum during the surface disinfestation period can also be helpful. Fungal and bacterial contamination of explants will be seen in 7 to 10 days and these are readily discarded.

Discarding visually contaminated explants should be considered only the first line of defense, as experience has shown that this is not an adequate test for cultures free of bacteria and fungi. Knauss and Miller (5) showed that there was Erwinia contamination in commercial tissue

cultures, which caused debilitation when the organism did not overgrow the culture. More recently I found a bacterial contaminant (Pseudomonas) in a commercial lot of broccoli cultures. It was difficult to observe contamination in the cultures. This contamination reduced the shoot multiplication rate to 1/3 of the bacterial-free cultures and virtually eliminated the ability of the cultures to root in Stage 3. Some contaminants may stay latent for several recultures before they become visually apparent. When this happens, after several recultures, it can cause a very serious interruption of the production system.

The method used in my laboratory to reduce this hazard has been to select vigorous and visually clean explants at the end of the first incubation period. During reculture, bits of the residue tissue are diced and placed in sterile culture containing 5 ml of Bacto nutrient broth. This culture is allowed to incubate alongside the Stage 2 subcultures for 10 days before ascertaining if latent disease potential exists. Even with using the indexing method we have experienced occasional latent disease escape. I have been told that other laboratories routinely add substances such as nutrient broth to their explanting media to assist in the identifying of explants contaminated with latent diseases. It would be helpful for a plant pathologist to completely assess the most efficient method of identifying explants with latent disease present.

There are several factors to be considered in assessing explant survival. Pre-plant conditioning is a subject area that is poorly understood but undoubtedly is a major factor in explant survival. I have experienced times when a complete lot of explants has died and then, with another attempt using the same stock plants, had a good survival rate. Some standard parameters on choosing stock plants should be to select healthy specimens and be sure their rest requirements have been satisfied. Stock plants that have been maintained under environmental conditions conducive for continued flushes of new shoots have been beneficial for producing adequate quantities of explant material and successful explants. This is done using a glasshouse equipped with supplemental light to satisfy daylength requirements for continual vegetative growth through the year. As our knowledge of the importance of pre-conditioning plants for explants increases, it may result in use of growth chambers with precise control of light quality, intensity and regulated temperature. All of the above factors influence plant metabolism and consequently affect the production of plant hormones that can either promote or inhibit plant growth.

The knowledge that juvenile plants are more readily explanted is only useful in limited situations for the plant propagator. Commercial plant tissue culturists want to propagate plant material that is of proven quality and this requires the propagation of plants that are in the adult stage of growth. Furthermore, it is undesirable to wait for several years until plants convert from the juvenile stage back to the adult stage of development. The principles learned by nurserymen about growth regulators and other cultural factors for successful propagation of diverse plant materials indicate that, with appropriate manipulations, organ initiation can be induced in many cases.

Medium composition is an area that experimentation can be readily done providing one has access to uniform explanting material. With rhododendrons and some other woody crops, the reduction of inorganics and sucrose to half-strength has been beneficial. The addition of growth regulators, auxins and cytokinins used in Stage 2 should be added in low concentrations as they are generally beneficial to explant health. Murashige has suggested the pre-soaking of explants in ascorbic and citric acid solutions and adding them to the culture medium to reduce tissue browning. Jones (4) added phloroglucinol to his medium and obtained increased shoot proliferation and rooting. Charcoal can be added to the medium to adsorb toxic substances released from the explant.

The physical condition of the medium is another important factor in explant survival. Sometimes explants are initially placed on a semi-solid medium. If the explants are susceptible to browning, this may aggravate the problem. Sometimes explant survival is improved with the use of the alternate method of incubating in a liquid medium. When using liquid media, one should compare stationary to slow agitation on a wheel revolving at 1 rpm. Other alternatives with liquid include placing the explant on a filter paper bridge or a synthetic support that is bathed in the nutrient medium. These variations provide for a wide range of gas exchange. Explants sometimes need frequent reculturing at 3-7 day intervals for survival. This will provide an escape mechanism for the explant from the toxic substances that are produced. Explants 2-5 cm long frequently have greater survival when placed with the base of the shoot bathed in a small amount of culture medium compared to placing the shoots on a semi-solid culture medium. The improved survival rate becomes apparent when the new shoots start growing actively, whereas explants on the semi-solid medium will initially start new growth but then suddenly stop and wither.

## STAGE 2: Propagule multiplication

This stage is very important simply because the multiplication rate is the major economic criterion for successful commercial tissue culture propagation. The major tissue culture objective in this stage is to produce the maximum number of useful propagule units in each reculture incubation. These propagules must be of adequate size in order to be physically separated with reasonable speed and the propagules produced need to be of a uniform size and capable of consistent production. The propagule multiplication of commercial tissue cultured fruit crops is at present through axillary and adventitious bud development. At this time the method of multiplication is not so much regulated by choice but rather through characteristic types of multiplication the crop is capable of producing. The other method of tissue culture multiplication, somatic embryogenesis, although having great multiplication potential, remains to be developed to a level of practical commercial usefulness.

Therefore, a strategy should be considered when starting work on a new crop to be sure a system has been perfected for producing the maximum number of useful propagules per reculture. A good starting place is to

first review the literature to determine what has already been accomplished. This may provide an outline on how to get started. One should realize that with the diversity of varieties in each fruit crop there may be fine differences in growth regulator requirements. This may require manipulation of the culture medium for producing the maximum numbers of useful shoots. The prudent tissue culturist will work out his system and run a commercial pilot study prior to setting his production goals and pricing.

Determination of the appropriate growth regulators and their concentrations is of major importance in developing the appropriate culture medium. The two major groups of plant hormones for consideration are cytokinins and auxins. These have a major control over organogenesis. The choice of the right hormone is fundamental for meeting the objective of maximum propagule production. Commercially available cytokinins include kinetin, N<sub>6</sub>-benzyladenine (BA) and N<sub>6</sub>-isopentenyladenine (2iP). The auxins used in axillary and adventitious proliferation are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthaleneacetic acid (NAA). The proper choice of cytokinin and auxin, and the adjustment of concentrations of the two hormones, is necessary for obtaining propagation of the maximum number of propagules per sub-culture.

There is a period of time required to build up stock cultures from the original explants to production quantities and this provides an opportunity for verifying the appropriate growth regulators and their concentrations. Once the stocks are increased sufficiently to provide 100 representative reculture samples, it is time to begin testing for the appropriate cytokinin. This is done by using 10 treatments with 10 replicate cultures consisting of a no cytokinin control, and 1, 5, and 10 mg per liter each of kinetin, BA and 2iP. The auxin is added to the basal medium and generally is 1 mg per liter of IAA. After an appropriate period of incubation, the number of propagules per culture along with the general health of the cultures can be evaluated. All of the plant material is saved and recultured on the best treatment found in the test to equilibrate the stocks on a constant medium.

The next test is to determine the auxin of choice. This again requires 10 treatments including a no auxin control, and 1, 2.5, and 5 mg per liter each of IAA, NAA and IBA. The best cytokinin and concentration treatment in the previous test is added to the basal medium. All of the extra stocks are recultured on the best medium available at that time.

The final test involves a factorial experiment combining the best auxin and cytokinin in a concentration range of each compound. This will require between 16 and 25 test treatments (range of 4-5 auxin and 4-5 cytokinin treatments in all possible combinations) to ascertain the appropriate concentration for producing the maximum number of usable propagules. The incubation period will vary with each crop and ranges from 5 to 10 weeks. Therefore, the time needed to obtain a complete

answer to the appropriate hormones and concentrations may range between 4 and 8 months. This should not be considered lost time since it is necessary to have about this much time to build the stock cultures to an appropriate size for production needs.

The choice of appropriate auxin and cytokinin is unlikely to change between cultivars. However, it is probable that the concentration of the growth regulators will vary between cultivars. Therefore, factorial growth regulator tests should be done on several diverse cultivars before concluding that one set of growth regulator concentrations is adequate for all cultivars. It is advisable to repeat and compare the better treatments for 2 to 3 consecutive subcultures to be certain the results are consistent with several incubations.

The identification of the appropriate inoculum and correct positioning on the culture medium are important factors to consider for maximum propagule production. The basal tissue mass is sometimes the appropriate inoculum for reculture in some systems. Other crops require reculturing of the crowns or the actively growing shoots for maximum shoot multiplication. It is a useful experiment to divide the cultures into the different morphological units in order to identify which units will produce the maximum propagule multiplication. Likewise, the position of the recultures on the medium is important. Some crops multiply only where the tissue is in contact with the medium.

The size of the reculture inoculum and the density of inoculum are also important factors for maximum propagule multiplication. A careful observer will recognize that the size of recultured inoculum has an influence on the length of recovery time after subculturing and, ultimately, the incubation time required to achieve maximum usable propagules. This indicates there is often a minimum critical mass when cutting tissue cultures for reculturing. Ignoring this will contribute to size variability in the propagules produced. The propagule production capability between cultivars will often vary. Sometimes this can be compensated for by altering the density of the inoculum planted on the new cultures. This is a fine-tuning method for controlling the number of propagules used per culture without altering the hormone concentrations of the medium.

The use of liquid medium has some attractive economic features over a semi-solid medium when proven to be a functional alternative. These include savings of the cost of agar and faster reculture rates since the technician is not concerned with precise orientation on the culture medium. At the present time it would appear economically infeasible to consider agitation of liquid cultures for mass propagation.

The appropriate time intervals between recultures should be systematically determined. After the ideal propagule has been determined under a specific set of circumstances, a test should be done to count the number of shoots produced with time. This, along with an observation of culture health, will readily determine the optimum incubation time and safe time interval for reculturing healthy propagules.

The choice of culture container should be based on how cultures grow. Cultures of blueberries adequately grow and multiply in 25 mm diameter culture tubes because they produce numerous fine stems with small leaves. Cultures of crops like strawberries require space to spread out and need larger diameter culture containers.

Definitive information on growth room environments is difficult to obtain since serious research requires several reasonably sophisticated environmental chambers to obtain reliable results. Many growth rooms have been arbitrarily set to operate with light sources of cool white and Gro-Lux fluorescent tubes with about 1,000 lux intensity on a 16-hour light, 8-hour dark cycle and temperatures set at a constant 25° to 27°C. It may be an advantage to have temperatures reduced to 20°C for some deciduous fruit crops.

Some fruit crops do not respond to their maximum potential if grown on Murashige and Skoog (MS) salts (11). Examples are red raspberries and blueberries, which are apparently affected by the general salt toxicity as shown by foliage chlorosis and browning of the stems, particularly when in contact with the medium. I found when working on rhododendrons that the survival of cultures was difficult on the MS formula. With a systematic study I found that it was necessary to modify this formula by reducing the ammonium nitrate and potassium nitrate to approximately 1/4 strength and adding twice the strength of ferrous sulfate and Na<sub>2</sub>EDTA (1, 2). These changes dramatically improved the propagule multiplication and culture health. Subsequently, the potassium iodide has been reduced as it was found to inhibit root initiation. An evaluation of the inorganic formulas for crops is suggested by comparing the MS and rhododendron inorganics. This should be done for 2 to 3 consecutive recultures with the propagule counts made at each incubation period.

### STAGE 3: Root initiation and hardening for transfer to soil

During the discussion on Stage 2 multiplication we dealt with factors that affect production of uniform propagules. If a good job has been done in producing uniform plant materials in Stage 2, there will be only minor adjustments necessary during the reculture in Stage 3. From a practical standpoint, the division of individual propagules provides another opportunity to assure maximum uniformity through discarding of non-uniform propagules. Those propagules that are chlorotic, watery in appearance, or obviously phenotypically abnormal should be routinely discarded. For maximum uniformity, the size range of propagules that will result in uniform growth and rooting should be determined.

The major objectives during Stage 3 are to condition and develop the propagules sufficiently to survive the stress conditions that will be experienced by transferring them to soil and to encourage initial rapid growth. Most propagules coming from Stage 2 have no roots and are susceptible to desiccation. Stage 3 should induce rooting and encourage

hardening of the plants as well as increase plant size. With some crops root induction is the most difficult factor to achieve. The following are some cultural factors that have been found beneficial for root initiation:

1. Low salt concentrations in the medium. The reduction of the inorganic salt concentrations of the MS and the rhododendron formula to  $1/4 \times$  have been beneficial in root induction. The reduction or elimination of potassium iodide from the nutrient formula was beneficial in the rooting of rhododendrons.
2. The growth regulator requirements are basically the addition of auxin. The appropriate auxin and concentration should be systematically determined.
3. The addition of adsorbents such as activated charcoal has been beneficial in the rooting of raspberries and blueberries. Jones (4) claims the addition of phloroglucinol has been beneficial in the rooting of apples.
4. Agar generally is used to support the propagules. The concentration should be as low as possible yet capable of supporting the plantlets.
5. Seibert (12) found root initiation to be stimulated by illumination with red light.

#### STAGE 4: Acclimatization of tissue cultured plantlets in soil

To date very little definitive research has been published on this very important transition stage. Both the environmental condition of the potting mix and the atmosphere must be considered. Kyte and Briggs (6) found that a porous potting soil of peat-perlite-composted bark 1:1:1 was the best for rooting tissue-cultured rhododendrons. The depth of soil was apparently important as the survival rate was much greater in 4" pots than was obtained in shallow trays.

The tissue cultured plantlets should be thoroughly washed to remove nutrient medium from the plantlets to inhibit microorganism growth. The plantlets are very susceptible to desiccation and, consequently, humidity must be kept high with the use of humidity tents and/or intermittent mist. Since there is a relationship between humidity and temperature, temperature must also be closely controlled.

Precise information is needed on the optimum environmental requirements for light intensity and duration, temperature and humidity before they can be assessed for survival, growth and uniformity. This type of information is needed to determine the economic advantages of climate controlled rooms for acclimatizing tissue cultured plants.

One final comment is related to attempting to modify the third stage or eliminate it completely. In some crops the third stage can be successfully eliminated, bypassing one costly step of the tissue culture process. However, the plantlet survival rate may decrease and variability in size and growth may increase.

## SUMMARY

1. Explanting of some woody fruit crops has been difficult. Alternate methods to use small shoot apices should be tried.
2. To maximize the yield of any tissue culture propagation system, one must be cognizant of a multitude of factors, including an optimized culture medium, care in using an appropriate inoculum of consistent density and reculturing at specified intervals.
3. Rooting and acclimatization of plantlets in soil continues to be a major problem. Some suggestions are made, but a completely definitive answer is still lacking. There is much need for more research in both Stages 3 and 4.

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## Strawberry Micropropagation

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In the same way as for other species (24), the original aim of strawberry in vitro culture was not clonal propagation, but rather to obtain varieties without viruses, mycoplasmas, fungi, bacteria, tarsonemes or nematodes, the spreading of which tends to be encouraged by the traditional vegetative method of propagation even in carefully managed nurseries. Research in this direction has been extremely successful (3, 23, 26, 33) and, in fact, in vitro culture of strawberry tip meristems (taken from heat-treated plants) seems to be the best way of obtaining disease-free plants (21). But research intended to resolve a particular problem can sometimes be exploited on a much wider scale. This has been the case of the in vitro culture of strawberries, which was initially used to obtain a limited number of disease-free mother plants, but which is now the fastest and most efficient method of propagating strawberries.

Several research workers certainly contributed to the progress that has been made (1, 30), but Boxus undoubtedly made a fundamental contribution when he announced his industrial method of in vitro strawberry propagation in 1974 (5). His method was based on the properties that had already been known for some time of two hormones: in the presence of benzyladenine (BA), the dormant buds of a vegetative apex are stimulated to grow and to elongate, as are those that form on the new axis; removing this hormone from the medium stops the process of bud self-reproduction and each bud forms roots and produces a complete new plant.

The method proposed by Boxus is based on the following phases and is shown in Fig. 1: a) sterile culture of tip meristems of different sizes; b) virus indexing; c) in vitro mass-propagation of shoots; d) root formation; e) transfer of the plantlets to open field culture.

As can be seen, there is little difference between this method and that adopted for other species, including ornamental plants (2, 8, 16, 19, 32). The method can be called mass-propagation insofar as it allows a practically infinite cyclical production of buds.

The basic cultural medium for the in vitro phases (a, c, d) is given in Table 1. The addition of hormones varies according to the phase (6).

In our work at the Rome Fruit Experimental Institute, we have replaced the glucose with 20 g/liter of sucrose since the explants are then greener and longer. The quantity of 8 g/liter of agar suggested by Boxus is only indicative since the quality of this product varies, but it must always give a very soft gel (Boxus, personal communication). The pH of the medium is adjusted before autoclaving with 0.1 N NaOH or 0.1 N KOH. For the medium for 'Aliso' we prefer to use KOH since this cultivar seems to grow better with more potassium (14).

Sterile culture of tip meristems of different sizes. All the buds of the plant can be used to begin the culture. Only because sterilization and handling are easier is it advisable to use young runner plants that have just started to root. The tip can be sterilized in various ways; in Rome, we use a 1.5% sodium hypochlorite water solution, taking care to rinse explants several times in sterile water. Very good results are also obtained with a water solution of calcium hypochlorite with 1% of free chlorine or with 0.5% merthiolate (17).

To the basic substrate must be added 1 mg/liter of indolebutyric acid (IBA), 0.1 mg/liter of BA and 0.1 mg/liter of gibberellic acid (GA<sub>3</sub>). This hormone balance is the best that we have tested as far as the growth of the meristem is concerned. If the initial explant is a tip larger than 0.5 mm, the hormone balance of phase (c) could well be used. To date we have not found any significant differences with incubation temperatures of between 20° and 28°C (unpublished results).

The photoperiod we use is 16 hr at 2,000 lux (c. 200 ft-c). After 45 days of culture in the case of a small tip meristem and 30 days for a larger one, we obtain a whole plantlet with a few roots and the typical unifoliate leaf of the juvenile phase.

Virus indexing. With in vitro culture of strawberries, it is very unlikely that parasitic fungi can spread; even if they are taken together with the apex, the cultures are rapidly contaminated and the explants destroyed. This is not the case with viruses, however, since explants attacked by a virus multiply equally well and there is a considerable risk of disseminating diseased plants. It is therefore essential that there should be a virus check. This is usually done by grafting the middle leaflet of a leaf of the plant to be indexed in place of a similar leaflet of a leaf of Fragaria vesca (4, 7, 22). To permit the indexing to be carried out a number of the plantlets produced are allowed to root and then grown in the greenhouse in order to obtain adult plants, while the others are kept in the refrigerator at 2°C.

In vitro mass propagation of shoots. At the conclusion of the indexing process, the disease-free plants are transferred to the following medium: the basic medium of Table 1 plus 1 mg/liter of BA, 1 mg/liter of IBA and 0.1 mg/liter of GA<sub>3</sub>. The temperature of the growth room is 22° to 25°C and the photoperiod 16 hr at 2,000 lux. As soon as the explant is put into this fresh medium, it starts to produce new shoots. Within 20 to 30 days, the number of new shoots produced is large enough to permit their division and transfer to the same fresh medium, a process that can be repeated ad infinitum. In a year, more than one million new plants can be produced from one shoot. Nonetheless, even though this medium leads to a generally adequate proliferation of shoots in all cultivars (Boxus, personal communication), the propagation efficiency, measured as the number of plants produced per day of subculture, varies from cultivar to cultivar. In the first place, it depends upon the amount of BA employed. Some cultivars, such as 'Tioga', 'Belrubi', 'Pocahontas', 'Gorella' and 'Sequoia', propagate rapidly producing shoots of good

quality with 1 mg/liter of BA. For others, such as 'Aliso', 'Primella', 'Rabunda' and 'Redgauntlet', it is necessary to reduce the quantity of BA, which, at 1 mg/liter, leads to poorly developed shoots, and, in short, to a general decline in the quality of the material and a smaller number of plants. The interaction of auxin and cytokinin has also been shown to be essential for normal development of explants: concentrations of 0.25 to 2.5 mM of cytokinin and 0.25 to 1.0 of auxin seem to give the best results (18). GA<sub>3</sub> seems to control the proliferation rate (29).

The source of nitrogen also has a considerable influence on propagation efficiency. In a recent experiment with 'Aliso' (15), the explants were unable to use (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as the only source of nitrogen. Indeed with concentrations higher than 3 mM, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> proved to be toxic. Nitrates were the only form of nitrogen to have given good results and the development and multiplication of shoots depended upon the quantity employed. In fact 'Aliso' gave its best results with 4 to 8 mM of NO<sub>3</sub> in addition to Knop's macroelements (14). Other authors suggest other changes in the growth conditions (20).

Root formation. The medium for the root formation phase contains 1 mg/liter of IBA but no BA or GA<sub>3</sub>. All the other growth conditions remain unchanged. As soon as the explants are put in the rooting medium the leaves become greener and the plantlets begin to elongate. Between the 10th and 12th day of incubation they start to form roots, which reach a length of 3 to 4 cm between the 25th and the 30th day. This medium can also be modified. In Rome, we have found that the addition of 1 to 2 g/liter of activated charcoal causes the roots to grow faster and to reach a length of 3 to 5 cm in 10 to 15 days (10). Later experiments (unpublished data) with charcoal, but without IBA, did not give equally satisfactory results.

Transfer of the plantlets to open field culture. With root formation the in vitro phase in the strict sense comes to an end.

The rooted plantlets are removed from the test tubes, thoroughly washed to remove the remains of the agar medium, and transferred to the greenhouse. In our experiments, the soil in which plants are most successfully transplanted has been found to be pure peat or a 1:1 mixture of sand and peat. The return to vegetative growth in the greenhouse is considerably influenced by the pH of the soil. The best results are obtained with peat which has a pH between 5.5 and 7.0 (unpublished data).

Another critical factor is relative humidity, which must never be less than 90%. It is necessary to maintain relative humidity at 90% for at least 15 days, covering the plants with plastic film. After this period the plastic can be removed and normal irrigation is enough to ensure steady growth. In order to allow rapid development, there must be a long photoperiod. By about the 45th day the plants have 3 to 5 trifoliate leaves of the adult phase, a well formed crown and a satisfactory root system. At this point they can be transferred to the open field (9).

Cold storage of in vitro material. The whole procedure outlined above can be scheduled, since in vitro explants can be stored for long periods at low temperatures (6, 11, 25, 29). The best results are obtained by cold storing explants that are in the budding medium and have reached maximum growth, i.e. after 10 to 15 days. Rooted plants can also be stored for several months (6).

Nursery runner plant production by means of micropropagated mother plants. The results of trials carried out in Italy have shown that there is a considerable difference between the nursery behavior of micropropagated mother plants and that of those produced by traditional methods (Tables 2 and 3). For the cultivars tested, both the number of runners and the number of runner plants produced have always been significantly larger for the micropropagated mother plants. The commercial classification of plants on the basis of their crown diameter shows that the number of both the first and second grades is larger for the micropropagated mother plants (Table 2). Nonetheless, both leaf surface area and the average of the crown diameter of all the plants produced reveal a tendency for the micropropagated mother plants to produce smaller runner plants (Table 3) (12).

Fruit production and behaviour of micropropagated plants. While the in vitro propagation of strawberries has proved to be an effective method for the production of a large number of mother plants to be used in nurseries and, consequently, for the rapid diffusion of new cultivars, it does not appear suitable at present for the direct supply of plants for fruit production.

Micropropagated plants transplanted during the summer have immediately produced runners in larger numbers than those grown by traditional methods. At the beginning of the winter they have a large central crown of flowers and in the following spring produce more flowers than traditional plants, but with shorter stems. This phenomenon is extremely marked in the case of 'Gorella', less so for other cultivars.

The crop from micropropagated plants has always been less than that of traditional plants.

It is extremely interesting, instead, to compare the production of plants produced from micropropagated mother plants with that of traditional plants (Tables 4 and 5). There is a clear tendency for plants derived from micropropagated mother plants to give heavier crops both from fresh and cold-stored plants in the open-field cultivation.

The crops and behavior of micropropagated plants found in the tests made so far suggest that further experiments are necessary, especially in connection with the period of planting (13).

Concluding remarks. The in vitro propagation of strawberries, in the same way as for other species (27), has advantages and disadvantages.

The most important advantage is that of being able to produce millions of disease free plants per year in a relatively small space. The method described does not allow virus infection to occur during the in vitro propagation phase. The test tubes containing the explants can be stored for a long time, thus making it possible to plan the activity of an industrial laboratory and the creation of an in vitro strawberry germplasm.

Furthermore, in vitro culture makes it possible to avoid the delay inherent in the period of quarantine imposed by a great many countries. These advantages are further increased when the tests designed to reveal viruses are properly carried out for the largest possible number of known viruses, when there are no doubts regarding the productivity of the clone or of the variety to be propagated and when the most effective measures are taken against insects and diseases both in acclimatization greenhouses and in nurseries. This is absolutely essential in view of the fact that in vitro culture does not build in any resistance (13, 31).

The greatest disadvantage is the high initial cost of a laboratory and the need to employ highly qualified staff. But if the economics of in vitro strawberry culture are to be judged by the number of laboratories that have been set up in the United Kingdom, the Netherlands, Belgium, France, Germany, Italy, and the United States, as well as those that will shortly start operating in Spain, Rumania, and Greece, it has to be admitted that these new techniques also have a bright future outside the ornamental field.

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Table 1. Composition of basal medium for strawberries.

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Macronutrients (Knop's) in mg/liter:

$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  - 1000;  $\text{KNO}_3$  - 250;  $\text{KH}_2\text{PO}_4$  - 250;  
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  - 250.

Micronutrients (Murashige and Skoog, 28) in mg/liter:

$\text{MnSO}_4 \cdot \text{H}_2\text{O}$  - 16.9;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  - 8.6;  $\text{H}_3\text{BO}_3$  - 6.2;  
 $\text{KI}$  - 0.83;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  - 0.025;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  - 0.025;  
 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  - 0.25.  
 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  - 27.8 plus  $\text{Na}_2\text{EDTA}$  - 37.2.

Vitamins (28) in mg/liter:

myo-inositol - 100; glycine - 2; nicotinic acid - 0.5;  
pyridoxine HCl - 0.5; thiamine - 0.1.

Sugar in g/liter:

glucose - 40

Agar - 8 g/liter

pH 5.6

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Table 2. Number of runner plants and size of runner plants (commercial grading) produced from standard or micropropagated mother plants.

| Cultivar and type | Runner plants per mother plant | Crown diameter      |                      |                    |                      |                    |                      |
|-------------------|--------------------------------|---------------------|----------------------|--------------------|----------------------|--------------------|----------------------|
|                   |                                | ≥8 mm               |                      | 6-7.9 mm           |                      | < 5.9              |                      |
|                   |                                | % of total          | no. per mother plant | % of total         | no. per mother plant | % of total         | no. per mother plant |
| Gorella           | microprop.                     | 62.0 <sup>**z</sup> | 71.7                 | 44.4 <sup>**</sup> | 15.7                 | 9.8 <sup>**</sup>  | 12.6                 |
|                   | standard                       | 39.3                | 79.9                 | 31.4               | 11.3                 | 4.4                | 8.8                  |
| Belrubí           | microprop.                     | 26.2 <sup>**</sup>  | 88.6                 | 23.3 <sup>**</sup> | 5.9                  | 1.5 <sup>**</sup>  | 5.6                  |
|                   | standard                       | 10.8 <sup>**</sup>  | 83.4                 | 9.0 <sup>**</sup>  | 8.7                  | 0.9 <sup>**</sup>  | 7.9                  |
| Aliso             | microprop.                     | 146.3 <sup>**</sup> | 54.2                 | 79.3 <sup>**</sup> | 15.3                 | 22.4 <sup>**</sup> | 30.5                 |
|                   | standard                       | 89.5 <sup>**</sup>  | 61.5                 | 55.0 <sup>**</sup> | 14.8                 | 13.2 <sup>**</sup> | 23.7                 |

<sup>z</sup> Statistical comparison of means: \*\* = significant at P = 0.01.

Table 3. Vegetative characteristics of runner plants produced from micropropagated and standard mother plants.

| Cultivar and type of plant | Leaf surface (cm <sup>2</sup> ) | Crown diameter (mm)  | Stem diameter (mm) | no. of runners per mother plant |
|----------------------------|---------------------------------|----------------------|--------------------|---------------------------------|
| Gorella                    | micropropagated                 | 40.44 <sup>**z</sup> | 12.1 <sup>**</sup> | 1.9 <sup>**</sup>               |
|                            | standard                        | 54.93                | 13.6 <sup>**</sup> | 2.3 <sup>**</sup>               |
| Belrubí                    | micropropagated                 | 52.96                | 15.6 <sup>**</sup> | 2.0                             |
|                            | standard                        | 49.80 <sup>ns</sup>  | 14.6 <sup>**</sup> | 2.1 <sup>ns</sup>               |
| Aliso                      | micropropagated                 | 23.84 <sup>**</sup>  | 7.6 <sup>ns</sup>  | 1.7 <sup>ns</sup>               |
|                            | standard                        | 29.10                | 8.4 <sup>ns</sup>  | 1.9 <sup>ns</sup>               |

<sup>z</sup> Statistical comparison of means: ns = not significant; \*\* = significant at P = 0.01.

Table 4. Fruit production and ripening data for 'Gorella' strawberry plants derived as runners from micropropagated or standard propagated mother plants when planted without (fresh) or with cold storage as compared to micropropagated mother plants direct from culture.

| Treatment and plant type | Production per plant (g) |            | Avg. weight of fruit (g) |            | Ripening precocity index |            | Date of full bloom |            |
|--------------------------|--------------------------|------------|--------------------------|------------|--------------------------|------------|--------------------|------------|
|                          | Plastic house            | Open field | Plastic house            | Open field | Plastic house            | Open field | Plastic house      | Open field |
| Runner Fresh             |                          |            |                          |            |                          |            |                    |            |
| micropropagated          | 377                      | 514 *      | 10.1                     | 8.8        | 23.4                     | 17.7       | 3/26               | 4/18       |
| standard                 | 448                      | 479        | 10.6                     | 11.1       | 21.3                     | 16.4       | 3/26               | 4/18       |
|                          | ns <sup>z</sup>          |            | ns                       | **         | ns                       | ns         |                    |            |
| Cold stored              |                          |            |                          |            |                          |            |                    |            |
| micropropagated          | 495                      | 512 *      | 13.2                     | 14.5       | 24.7                     | 20.4       | 3/31               | 4/28       |
| standard                 | 417                      | 435        | 14.7                     | 14.7       | 22.7                     | 17.8       | 3/29               | 4/28       |
|                          | ns                       |            | ns                       | ns         | **                       | **         |                    |            |
| Mother Fresh             |                          |            |                          |            |                          |            |                    |            |
| micropropagated          | 207                      | 338        | 9.2                      | 8.8        | 22.1                     | 15.0       | 3/25               | 4/18       |

<sup>z</sup> Statistical comparison of means: ns = not significant; \* = significant at P = 0.05; \*\* = significant at P = 0.01.

Table 5. Fruit production and ripening data for 'Aliso' strawberry plants grown in plastic tunnels. Runner plants were derived from micropropagated or standard propagated mother plants and planted without (fresh) or with cold storage and mother plants were planted in tunnels directly after acclimatization from tissue culture.

| Treatment and type of plant | Production per plant (g)      | Average weight of fruit (g) | Ripening precocity index | Date of full bloom | Col-lapsed plants |
|-----------------------------|-------------------------------|-----------------------------|--------------------------|--------------------|-------------------|
| <u>Runner</u>               |                               |                             |                          |                    |                   |
| <u>Fresh</u>                |                               |                             |                          |                    |                   |
| micropropagated             | 657 <sub>ns<sup>z</sup></sub> | 10.0 <sub>ns</sub>          | 18.0 <sub>ns</sub>       | 2/10               | 0                 |
| standard                    | 650                           | 10.4                        | 16.4                     | 2/12               | 0                 |
| Cold stored standard        | 725                           | 10.0                        | 15.5                     | 2/19               | 0                 |
| <u>Mother</u>               |                               |                             |                          |                    |                   |
| <u>Fresh</u>                |                               |                             |                          |                    |                   |
| micropropagated             | 617                           | 11.0                        | 16.2                     | 2/10               | 0                 |

<sup>z</sup> Statistical comparison of means: ns = not significant.

# Strawberry large-scale propagation

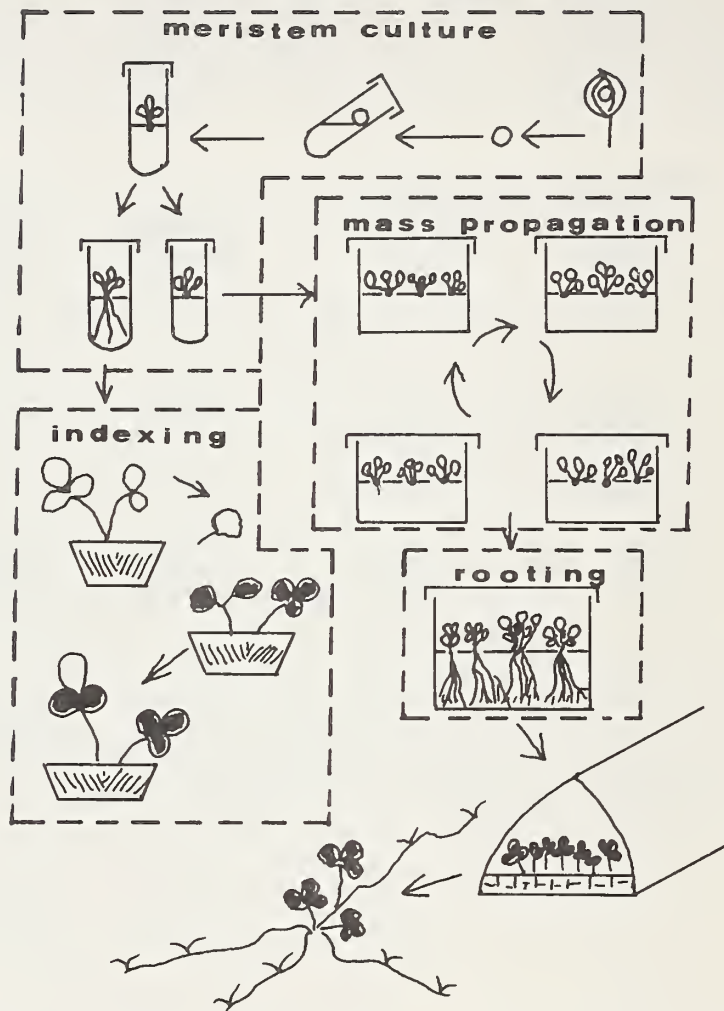


Figure 1. Schematic outline of phases involved in large-scale propagation of strawberries from meristem-tips.

## Micropropagation of Thornless Blackberries

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The USDA small fruit breeding program has developed several genetically thornless blackberry cultivars in recent years. The standard propagation techniques of tip layering and rooting of stem cuttings were reputedly inadequate to produce sufficient plants to meet the demand for these cultivars. Thus, we set out to develop a rapid in vitro propagation technique for these cultivars.

As we were working only with apples at the time, we began by trying the apple medium of Jones (2) which we were then using. It soon became evident that the phloroglucinol used in Jones' medium was not necessary for the thornless blackberries (1). The resulting medium thus was the Linsmaier and Skoog modification (3) of the Murashige and Skoog high-salt medium (4) (Table 1). All components are now autoclaved since filter sterilization of the indolebutyric acid (IBA), as originally done, proved to be unnecessary.

For the establishment of cultures, actively growing shoot tips 4 to 5 cm long are collected. The leaves are removed except the 2 or 3 youngest ones enclosing the shoot apex and the shoot tips are dropped into distilled water containing 1 drop of liquid Micro detergent per 100 ml. After agitating gently for about a minute, the shoot tips are rinsed for 5 minutes each in several changes of distilled water, again with gentle agitation. In a transfer hood, a solution of 0.26% sodium hypochlorite (Clorox bleach diluted 1:20) plus 0.01% Tween 20 is added to the shoots and they are agitated for 5 minutes. This solution is poured off and the shoots are rinsed in sterile distilled water, first briefly for 3 changes and then for 5 minutes. The shoot tips are trimmed to a length of 1 to 2 cm and aseptically transferred to 15 ml of liquid medium in a 125 ml Erlenmeyer flask sealed with aluminum foil. The flasks are rotated at 1 rpm for 3 weeks. Light is provided 16 hr per day by high output fluorescent lamps at about 25°C.

For proliferation, the shoots are transferred to a solid medium of the same nutrient composition. The stem pieces are placed horizontally on the medium, after some leaves are trimmed off, to insure adequate contact between the stem and the medium. Subsequent subcultures are made at 3 to 4 week intervals. Cultures are grown at 24° to 26°C with 16-hour photoperiods at a light intensity of 2,000 to 4,000 lux from warm white fluorescent lights.

During the proliferation stage, axillary buds grow out from the original explant and lateral shoots develop on many of these newly produced shoots so that a dense mass of shoots is produced. These shoots

can be cut back to stimulate further shoot production during subculturing. The excised shoots can be used to establish further cultures or can be used as cuttings to be rooted.

Although cuttings can be rooted on agar medium, we find that it is simpler and easier to root them directly under mist or in high humidity under plastic. Cuttings with a stem length of 1 to 2 cm and having several leaves are collected and inserted into the rooting medium. Finely milled sphagnum makes a good rooting medium but cuttings can also be rooted in peat pellets (Jiffy-7) or other media. Cuttings treated with an auxin (e.g. 0.1% IBA on talc or Rootone F) root better than 90% in 4 weeks (1). Rooting is nearly as good without auxin treatment (Table 2) but differences occur among cultivars. The results shown here for tissue-cultured cuttings closely parallel results obtained in our laboratory with one-node softwood cuttings of the same cultivars and selections (5).

Rooted cuttings are easily acclimated to greenhouse conditions by reducing gradually the amount of mist applied or the humidity in the rooting chamber. The rooted cuttings can then be potted and grown in the greenhouse. They attain sufficient size for field planting in 5 to 7 weeks. We have found that 7.5 cm (3 inch) peat pots filled with a mixture of 1 part soil and 1 part peat-vermiculite mix are very good for growing these plants to field planting size.

For maximum production, the mother cultures are transferred to fresh medium after each batch of cuttings has been harvested. Care is taken to remove any callus that might form on the stems and any dead or discolored tissue. In this way, the mother cultures can be kept in continuous production for many months.

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Table 1. Composition of medium used for establishment and proliferation of thornless blackberry cultivars.

| Component   | Amount per liter |        |
|---|------------------|--------|
|   | mmol             | mg     |
| NH <sub>4</sub> NO <sub>3</sub>                     | 20.6             | 1650   |
| KNO <sub>3</sub>                                    | 18.8             | 1900   |
| CaCl <sub>2</sub> ·2H <sub>2</sub> O                | 3.0              | 440    |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O                | 1.5              | 370    |
| KH <sub>2</sub> PO <sub>4</sub>                     | 1.2              | 170    |
| FeSO <sub>4</sub> ·7H <sub>2</sub> O                | 0.1              | 27.8   |
| Na <sub>2</sub> EDTA                                | 0.1              | 37.2   |
| MnSO <sub>4</sub> ·H <sub>2</sub> O                 | 0.1              | 16.9   |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                | 0.03             | 8.6    |
| H <sub>3</sub> BO <sub>3</sub>                      | 0.1              | 6.2    |
| KI  | 5.0 $\mu$ mol    | 0.83   |
| Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O | 1.0 $\mu$ mol    | 0.25   |
| CoCl <sub>2</sub> ·6H <sub>2</sub> O                | 0.1 $\mu$ mol    | 0.025  |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                | 0.1 $\mu$ mol    | 0.025  |
| myo-inositol  | 0.56             | 100    |
| Thiamine HCl  | 1.2 $\mu$ mol    | 0.4    |
| Benzyladenine (BA)                                  | 4.44 $\mu$ mol   | 1.0    |
| Indolebutyric acid (IBA)                            | 0.49 $\mu$ mol   | 0.1    |
| Gibberellic acid (GA <sub>3</sub> )                 | 1.30 $\mu$ mol   | 0.5    |
| Sucrose   | 87.6             | 30,000 |
| Agar (when used)                                    | ---              | 5,000  |
| Adjust pH to 5.2                                    |                  |        |

Table 2. Rooting response of cuttings of thornless blackberry cultivars rooted under plastic with no auxin treatment.

| Cultivar or<br>selection | % Rooting<br>Subculture <sup>z</sup> |    |    | $\bar{x}$ |
|--------------------------|--------------------------------------|----|----|-----------|
|                          | 5                                    | 6  | 7  |           |
| Black Satin              | 92 <sup>y</sup>                      | 90 | 90 | 91        |
| Dirksen Thornless        | 84                                   | 90 | 60 | 78        |
| Smoothstem               | 85                                   | 64 | 67 | 72        |
| Thornfree                | 94                                   | 83 | 82 | 87        |
| SI-US 68-6-6             | 86                                   | 92 | 81 | 86        |
| SI-US 68-6-17            | 94                                   | 86 | 90 | 90        |
| $\bar{x}$                | 89                                   | 84 | 78 |           |

<sup>z</sup> Rooting response evaluated after 38 days for subculture(S) 5, 37 days for S-6 and 22 days for S-7.

<sup>y</sup> Percentages based on 200 cuttings per cultivar for each subculture.

# Tissue Culture Propagation of Red Raspberries

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Abstract. A two-fold increase in shoot multiplication was achieved with five varieties of red raspberry on three consecutive incubations on Anderson inorganic salts when compared to the Murashige and Skoog inorganic salts. The optimal concentrations of hormones for shoot multiplication of red raspberry were 0.03-0.5 mg/liter indolebutyric acid (IBA) and 1-2 mg/liter benzyladenine. In vitro rooting of red raspberries was successful using the basal medium that included Anderson's inorganics, 600 mg/liter activated charcoal and 0.2-1.6 mg/liter IBA.

## Introduction

Commercial tissue culture propagation of crops in the genus *Rubus* has several important applications. The primary one is in the multiplication of those crops that are traditionally propagated through shoot tip layering. The layering method is costly and frequently results in disease infection of the daughter plants. Tissue culture propagation can be cost effective and produce plants free of diseases and pests. Red raspberry can be efficiently propagated through suckers arising from roots and it is unlikely that commercial tissue culture propagation can be cost effective for large scale production. Therefore, the usefulness of tissue culture of red raspberries may be limited to rapidly increasing 1) new stocks to commercially usable quantities, 2) the maintenance of germplasm, and 3) increasing disease-free planting stocks.

Shchelkunova (12) used etiolated shoot tips of red raspberry to study the seasonal effects of explanting on the rate of successful cultures established. He found that March through June was the optimal time for explanting red raspberry and that indolebutyric acid (IBA) was the most suitable auxin to use in the culture medium. Jennings (9) reported that 0.5 mg/liter N-6-benzylaminopurine (BA) was the most effective concentration in the propagation of three varieties of red raspberries.

Broome and Zimmerman (4) utilized a medium containing Murashige and Skoog (MS) (11) inorganics, 1 mg/liter IBA and 1 mg/liter BA for successful propagation of thornless blackberry. They did not report the effects of varying hormonal concentration on shoot multiplication rates. Rooting of the shoots in cultures was accomplished through the elimination of BA and maintaining the IBA concentration in the culture medium. Their maximum rooting was 64%. Harper (6) reported successful explanting and rooting of blackberry and tayberry using a culture medium containing MS inorganics and IBA. Huth (7), attempting to obtain virus-free red raspberry plants through explanting axillary buds, utilized a medium containing MS inorganics, IBA, BA, and gibberellic acid.

James et al. (8) tissue culture propagated 12 raspberry seedling selections and 'Malling Jewel' using the Linsmaier and Skoog (10) medium supplemented with 1 mg/liter each of BA and IBA. They claimed the addition of 162 mg/liter phloroglucinol increased shoot multiplication and was also beneficial in the rooting medium.

Murashige and Skoog (11) developed an inorganic formula for maximum growth of tobacco callus that proved to be an effective inorganic formula for tissue culture propagation of many plants. Linsmaier and Skoog (10) evaluated organic amendments associated with MS salts and found 100 mg i-inositol and 0.4 mg thiamine HCl per liter were all the necessary vitamins for optimum growth of tobacco callus. They also reported that amino acids were not necessary for optimum callus growth. Gamborg et al. (5) in their guideline for developing tissue culture media, stated "a minimum of three subcultures with quantitative growth measurements against a standard medium is necessary to determine merit".

To date, systematic studies of hormonal concentrations to determine optimum multiplication rates of adventitious shoots have not been reported for specific *Rubus* crops. This paper reports the systematic study of IBA and BA concentrations for maximum shoot multiplication. The MS inorganics were compared with the Anderson inorganics that were originally designed for rhododendron shoot multiplication (1). Also the results of altering the hormonal concentration and addition of activated charcoal to the rooting media are reported.

### Materials and Methods

Shoot tip explants of red raspberry cultivars were obtained from cold-stored plants and from potted plants maintained in the greenhouse. The roots were removed, washed in soapy water and placed in quart jars along with moistened paper towelling. The jars were closed with loosely screwed jar lids and placed in darkness at 20-22°C for 10 to 14 days. Etiolated shoots arising from the root nodules were excised and surface disinfested 6-8 minutes in 0.53% sodium hypochlorite with a trace of Tween 20 surfactant added. The hypochlorite reaction was slowed down with a rinse of 0.05% sodium hypochlorite. Shoot apices were aseptically cut to 2-4 mm length and placed on the culture medium. New growth developed from the apical and axillary buds of the excised shoot tips.

All cultivars tested for hormonal concentrations were cultured for a minimum of four incubation cycles of 5-6 weeks each. This allowed the shoot multiplication rates to stabilize before utilizing the plant material. Small clumps of 3-5 shoots were recultured to maintain stock cultures.

Culture room temperature was a constant 21°C, with 18 hours of light per 24 hour cycle. The light source was cool white fluorescent light, with an average intensity of 20 uE/m<sup>2</sup>/sec.

The basal culture medium for explanting and shoot multiplication consisted of Anderson's inorganics (Table 1) developed for rhododendron propagation and the following organics in mg/liter: 30,000 sucrose, 100 myo-inositol, 0.4 thiamine HCl, 80 adenine sulfate dihydrate, 6,000 pre-washed agar. Adenine sulfate dihydrate was routinely eliminated from the basal rooting medium. The activated charcoal used in these experiments was obtained from the Grand Island Biological Company (GIBCO), 3175 Staley Road, Grand Island, NY 14072. All constituents in the culture medium were included and the pH was adjusted to 5.7 prior to autoclaving for 15 minutes at 121°C and 1.1 kg/cm<sup>2</sup>. Twenty ml of medium was dispensed in 25 x 150 mm culture tubes and closed with KaPuts. The hormonal concentrations routinely used for stock culture maintenance was 0.1 mg IBA and 4 mg BA per liter.

Test propagules were selected for uniform size and single shoots were planted perpendicular to the culture medium surface. The new shoot multiplication arose from the adventitious budding on the stem. Rate of shoot multiplication was determined by counting the number of shoots 3 mm long or longer produced per propagule after six weeks incubation. Each treatment was replicated 10 times. Standard error of the mean (S.E.) was calculated for all treatment means to provide a measure of variation between replicate cultures (13). The rooting index used was based on 1 = no roots; 2 = initial rooting to a few long roots; and 3 = many long roots with lateral branching.

## Results

The comparison of the MS inorganics to the Anderson inorganics is summarized in Table 2. Uniform stock from each of the five cultivars was divided and planted to fresh media containing the test inorganics. Shoot multiplication for the first incubation was lower than for the second and third consecutive cycles. In each incubation the best plant material was recultured back to the same treatment and consistently showed approximately twice as many usable shoots produced per propagule planted on the Anderson inorganics. Comparing the usable shoots produced indicates that multiplication rates will vary among cultivars.

The determination of appropriate hormonal balance was tested on two red raspberry cultivars. 'Heritage' was initially tested in a factorial experiment using 0, 0.5, 1 and 2 mg/liter IBA and 0, 2, 4 and 8 mg/liter BA (Table 3). The maximum number of shoots produced was 5.3 in the treatment containing 0.5 mg IBA and 2 mg BA per liter. The spread in treatment concentrations indicated that a lower concentration of IBA may increase multiplication rates. Subsequent experiments using 'Willamette' were carried out to test the use of lower concentrations of IBA. Results with 'Willamette' indicated 0.1 mg/liter IBA and 2 mg/liter BA were optimum for shoot multiplication of this cultivar. A subsequent series of experiments maintaining BA at 2 mg/liter with 2 consecutive incubations with varying concentrations of IBA showed a positive response with the addition of only 0.03 mg/liter IBA in the second incubation.

Preliminary experiments on rooting raspberries in culture indicated a narrow concentration range for positive auxin response and also positive effects with activated charcoal. An experiment was designed to test both the auxin and charcoal response for red raspberries. 'Willamette' rooted readily when 600 mg/liter activated charcoal was included in the medium (Table 4). The addition of IBA had no particular advantage on the percent of rooting or rooting index. However, the total plant growth was best with a range of IBA concentrations in combination with activated charcoal.

### Discussion

The comparison experiments of the MS inorganics to the Anderson inorganics were followed for three consecutive incubations as recommended by Gamborg et al. (5). The doubling of the usable number of shoots produced per incubation indicates the usefulness of Anderson inorganics for tissue culture propagation of *Rubus*. A comparison of the inorganics showed a reduction to approximately 1/4 strength of  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  as compared to the MS formula. Raspberry shoots grown on the MS formula tend to have a chlorotic appearance that could be a general salt toxicity.

James et al. (8) successfully propagated red raspberry with 1 mg/liter IBA and 1 mg/liter BA. In contrast, these results indicate the optimum culture medium to contain a lower range of IBA of 0.03-0.5 mg/liter. Results presented indicate that the maximum shoot multiplication rates can vary among cultivars (Table 2) and the optimum hormonal concentrations may also vary with cultivar (Tables 3-5). Factorial experiments may be advisable prior to propagating large quantities of any given cultivar using the following concentration ranges: 0-1 mg/liter IBA and 0-2 mg/liter BA.

In vitro rooting is essential for uniform plantlet production through the tissue culture system and transplant survival. Activated charcoal, 600 mg/liter, was essential for high rooting percentages. Red raspberries did not require the addition of auxin for root initiation, however, IBA improved the general growth of the plantlets.

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Table 1. Comparative analysis between the concentrations of inorganic compounds of Murashige and Skoog's 1962 and Anderson's 1978 formulas.

| Salts<br>macronutrients                             | MS       |      | Anderson |      |
|---|----------|------|----------|------|
|   | mg/liter | mM   | mg/liter | mM   |
| NH <sub>4</sub> NO <sub>3</sub>                     | 1650     | 20.6 | 400      | 5.0  |
| KNO <sub>3</sub>                                    | 1900     | 18.8 | 480      | 4.7  |
| CaCl <sub>2</sub> ·2H <sub>2</sub> O                | 440      | 3.0  | 440      | 3.0  |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O                | 370      | 1.5  | 370      | 1.5  |
| KH <sub>2</sub> PO <sub>4</sub>                     | 170      | 1.25 | --       | --   |
| NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O  | --       | --   | 380      | 2.75 |
| micronutrients                                      | mg/liter | μM   | mg/liter | μM   |
|   |          |      |          |      |
| KI  | 0.83     | 5.0  | 0.30     | 1.8  |
| H <sub>3</sub> BO <sub>3</sub>                      | 6.2      | 100  | 6.2      | 100  |
| MnSO <sub>4</sub> ·4H <sub>2</sub> O                | 22.3     | 100  | --       | --   |
| MnSO <sub>4</sub> ·H <sub>2</sub> O                 | --       | --   | 16.9     | 100  |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                | 8.6      | 30   | 8.6      | 30   |
| Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O | 0.25     | 1.0  | 0.25     | 1.0  |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                | 0.025    | 0.1  | 0.025    | 0.1  |
| CoCl <sub>2</sub> ·6H <sub>2</sub> O                | 0.025    | 0.1  | 0.025    | 0.1  |
| Na <sub>2</sub> EDTA                                | 37.3     | 100  | 74.5     | 200  |
| FeSO <sub>4</sub> ·7H <sub>2</sub> O                | 27.8     | 100  | 55.7     | 200  |

Table 2. Shoot multiplication rates per six-week incubation for three consecutive incubations on MS and Anderson inorganics. Hormone concentrations were 0.1 mg IBA and 4 mg BA per liter.

| Cultivar   | Inorganic formula | Shoot multiplication $\pm$ S.E.<br>Incubation |                |                |
|------------|-------------------|---|----------------|----------------|
|            |                   | 1   | 2              | 3              |
| Willamette | MS                | 2.1 $\pm$ 0.2                                 | 2.2 $\pm$ 0.4  | 2.4 $\pm$ 0.3  |
|            | Anderson          | 3.7 $\pm$ 0.7                                 | 7.7 $\pm$ 0.8  | 7.7 $\pm$ 0.7  |
| Heritage   | MS                | 2.6 $\pm$ 0.6                                 | 5.0 $\pm$ 0.8  | 4.5 $\pm$ 0.7  |
|            | Anderson          | 5.4 $\pm$ 0.9                                 | 9.2 $\pm$ 0.4  | 9.1 $\pm$ 0.5  |
| Skeena     | MS                | 1.6 $\pm$ 0.3                                 | 3.8 $\pm$ 0.8  | 3.5 $\pm$ 0.4  |
|            | Anderson          | 2.9 $\pm$ 0.3                                 | 5.8 $\pm$ 0.7  | 6.0 $\pm$ 0.6  |
| Nootka     | MS                | 2.4 $\pm$ 0.5                                 | 6.5 $\pm$ 0.6  | 7.0 $\pm$ 0.6  |
|            | Anderson          | 10.2 $\pm$ 1.5                                | 15.5 $\pm$ 0.2 | 14.3 $\pm$ 0.8 |
| Chilcotin  | MS                | 2.1 $\pm$ 0.5                                 | 3.0 $\pm$ 0.5  | 2.9 $\pm$ 0.5  |
|            | Anderson          | 5.3 $\pm$ 0.6                                 | 5.5 $\pm$ 0.6  | 5.7 $\pm$ 0.6  |
|            | MS                | 2.2   | 4.1            | 4.1            |
|            | Anderson          | 5.5   | 8.7            | 8.6            |

Table 3. Effect of IBA and BA concentrations on shoot multiplication rates of 'Heritage' and 'Willamette' red raspberries incubated 6 weeks.

| Cultivar   | IBA<br>(mg/liter) | Number of shoots $\pm$ S.E.<br>BA (mg/liter) |               |               |               |
|------------|-------------------|--|---------------|---------------|---------------|
|            |                   | 0  | 2             | 4             | 8             |
| Heritage   | 0                 | 0.8 $\pm$ 0.1                                | 4.1 $\pm$ 0.7 | 5.0 $\pm$ 0.9 | 1.8 $\pm$ 0.4 |
|            | 0.5               | 0.9 $\pm$ 0.1                                | 5.3 $\pm$ 0.1 | 2.5 $\pm$ 0.5 | 2.6 $\pm$ 0.5 |
|            | 1.0               | 0.9 $\pm$ 0.1                                | 2.8 $\pm$ 1.1 | 3.7 $\pm$ 0.8 | 1.3 $\pm$ 0.6 |
|            | 2.0               | 0.8 $\pm$ 0.8                                | 1.2 $\pm$ 0.2 | 3.5 $\pm$ 0.4 | 1.8 $\pm$ 0.3 |
| Willamette | 0                 | 1.1 $\pm$ 0.1                                | 3.1 $\pm$ 0.7 | 2.3 $\pm$ 0.5 | 0.8 $\pm$ 0.2 |
|            | 0.1               | 1.7 $\pm$ 0.3                                | 3.5 $\pm$ 0.6 | 0.9 $\pm$ 0.4 | 0.3 $\pm$ 0.2 |
|            | 0.5               | 1.2 $\pm$ 0.2                                | 2.8 $\pm$ 0.6 | 0.7 $\pm$ 0.3 | 0.9 $\pm$ 0.3 |
|            | 1.0               | 1.4 $\pm$ 0.2                                | 1.7 $\pm$ 0.5 | 2.1 $\pm$ 0.5 | 0.7 $\pm$ 0.3 |

Table 4. Effect of IBA concentrations and activated charcoal on rooting and plantlet growth of 'Willamette' red raspberry.

| IBA<br>(mg/liter) | Rooting<br>(%)   |     | Rooting<br>index |     | Fresh<br>wt<br>(mg) |          |
|-------------------|------------------|-----|------------------|-----|---------------------|----------|
|                   | -AC <sup>z</sup> | +AC | -AC              | +AC | -AC                 | +AC      |
| 0                 | 71               | 100 | 1.7              | 2.3 | 58 + 5              | 73 + 6   |
| 0.1               | 84               | 88  | 1.8              | 2.1 | 69 ± 6              | 83 ± 11  |
| 0.2               | 58               | 100 | 1.6              | 2.2 | 69 ± 9              | 104 ± 12 |
| 0.4               | 67               | 100 | 1.7              | 2.1 | 71 ± 9              | 93 ± 10  |
| 0.8               | 40               | 96  | 1.4              | 2.1 | 41 ± 5              | 90 ± 11  |
| 1.6               | 15               | 100 | 1.2              | 2.1 | 33 ± 4              | 99 ± 9   |

<sup>z</sup> AC = activated charcoal

Table 5. Red raspberry tissue culture propagation formulas.

| Constituent                           | Milligrams per liter             |         |
|---------------------------------------|----------------------------------|---------|
|                                       | Explanting and<br>multiplication | Rooting |
| Sucrose                               | 30,000                           | 30,000  |
| Anderson's inorganics (concentration) | 1 X                              | 1 X     |
| I-inositol                            | 100                              | 100     |
| Adenine sulfate dihydrate             | 80                               | --      |
| Thiamine HCl                          | 0.4                              | 0.4     |
| Indolebutyric acid (IBA)              | 0.1 (0.05-0.5)                   | 1.0     |
| Benzyladenine (BA)                    | 2 (1-2)                          | --      |
| Activated charcoal (Source GIBCO)     | --                               | 600     |
| Agar                                  | 6,000                            | 6,000   |
| pH (adjusted prior to autoclaving)    | 5.7                              | 5.7     |

# In Vitro Propagation of Grape<sup>1</sup>

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Reports concerning the propagation of Vitis species by tissue culture methods are rare in the scientific and technical literature. Currently, there is one report which deals with vine production from fragmented shoot apices (1) and two reports which demonstrate the feasibility of producing vines via somatic embryogenesis (4, 5). It is surprising that few papers exist, especially since grape is one of the world's largest fruit crops. Requirements for grafting and the ease with which many cultivars root may make grape a less desirable crop to explore. It is also possible that grape propagation by shoot tip multiplication may require little or no modification of established methods (6) and is therefore not reported.

The propagation of grape by shoot tip multiplication has several hypothetical advantages relative to conventional methods. Among these are the rapid increase of new hybrids, mutants and superior field plants and maintenance and production of virus-indexed foundation plants. It is not possible to determine whether cell culture methods are more advantageous in cases of abundant foundation material since cost comparisons do not exist.

The production of vines from isolated cells via somatic embryogenesis is essential for progress in anticipated genetic modification and engineering strategies for vine improvement. Such strategies may involve manipulation and selection of cells tolerant to physical (cold, heat, osmotic) and chemical (herbicide, bacterial and fungal toxins) stress. The use of somatic embryogenesis as an alternative method for the massive production of vines has great possibilities but methods for most cultivars have yet to be developed.

In this report the current progress in grape propagation by modified shoot tip cultures and somatic embryogenesis is reviewed.

Shoot Tip Culture. Barlass and Skene (1) developed a modified shoot tip culture method which involves the production of adventitious shoots from a fragmented shoot apex. These adventive shoots can then be multiplied by conventional shoot tip methods or rooted directly. The vines so produced assume juvenile leaf shape and tendril characteristics. It is not known if shoot tips produced by the release of repressed buds (conventional method) display juvenile characteristics.

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<sup>1</sup> Journal Article Number 1932 Rhode Island Agricultural Experimental Station.

<sup>2</sup> Part of this work is taken from a Thesis submitted by the junior author in partial fulfillment of the requirements for a MS degree.

The Barlass-Skene method (1) is as follows:

Mother Plant: 'Cabernet Sauvignon', rooted hardwood cutting, 4 months old, grown in greenhouse.

Explant: 10 mm shoot tip.

Sterilization: 5% (w/v) filtered calcium hypochlorite plus 0.01% Tween 20 for 15 min.; 3 rinses sterile distilled water.

Explant Preparation: Shoot apex, 1 mm long, 2 to 3 leaf primordia, cut into fragments, and teased apart with needles in petri dish with 5 ml medium. Ca. 20 fragments/apex.

Culture Medium: Murashige-Skoog (7) with 2 mg/liter benzyladenine, pH not given.

Culture Vessel: 50 mm diameter plastic petri dish, 5 ml liquid medium, sealed with parafilm.

Culture Environment: Cool white fluorescent ( $50 \mu\text{E}/\text{m}^2/\text{sec}$ ), 15 hr light, 9 hr dark. Temperature - 27°C light, 20°C dark.

Culture Schedule: 30 days - 90% of fragments form swollen leaf-like structures (10 mm long) and are transferred to 125 ml flask with same medium plus 0.6% agar (can move within 10 days with no change in productivity). 60 days - explant 30 mm long, shoots appear on basal swelling. Basal 50 mm<sup>2</sup> is excised (ca. 25 buds) and recultured on the same medium. 75 days - divide and reculture, repeat at 10 to 14 day intervals.

Root Initiation: Shoots longer than 3 mm on Whites (11) medium with chelated Fe and 0.6% agar. No growth regulators. Roots appear in 7 days.

Acclimation to Real World: Rooted cutting (21 days after excision) potted in Jiffy-7 peat blocks, covered with beaker for 9 days. Moved to pots with soil-perlite (40:60). Total elapsed time fragmentation to potting = 4 months.

Theoretical Yield: 1 apex = 8,000 vines in 3 to 4 months (with two subcultures).

Genetic Stability: Normal diploid plants, chromosome number ( $2n = 38$ ).

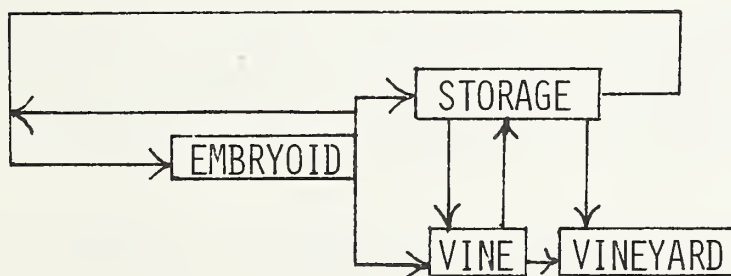
Somatic Embryogenesis: The production of embryonic structures (identical to those in seeds) from isolated plant cells was described by Steward et al. (9) and Reinert (8) in the late 1950's. Nearly 20 years later the formation of somatic embryos in grape callus was observed by Mullins and Srinivasan (5) and Krul and Worley (4). Embryoids of grape and other plants arise from proembryonic masses (PEMS) embedded in callus cells (4, 5). The factors involved in the initiation of PEMS are not

known. The transformation of PEMS to embryoids in grape occurs when they are shifted from a medium containing a strong auxin to one containing a weaker one (4) or when the cytokinin level of the medium is lowered (5). We have isolated a highly embryogenic callus of 'Seyval' which does not require growth regulators for growth. Embryoid formation in this callus occurs when it is grown on auxin for a short while and then cultured on auxin-free media, when the calcium concentration is one half that of Murashige-Skoog formulation, or when cultures are grown for long intervals without subculture. Most of the vines regenerated from the original 'Seyval' callus appear to be normal. They are currently being evaluated in the Montbray vineyards in Silver Run, Maryland, by Dr. Hamilton Mowbray.

A few primary embryoids displayed a tendency to produce embryoids at the transition zone of the root and hypocotyl (4). The formation of secondary embryoids in this manner was also observed in Eschscholzia (2), Ranunculus (3) and Brassica (10).

The development of normal vines from etiolated secondary grape embryoids is complicated by separate "dormancies" of the roots, hypocotyl, cotyledons and shoot apices each of which respond to different physical and chemical stimuli. The processes of embryoid production and of normal vine development appear to be mutually antagonistic. For instance, embryoids which produce embryoids generally senesce and die shortly after embryoid formation. In contrast, embryoids which develop flattened, green separate cotyledons or normal shoots generally lose the capacity for production of secondary embryoids. Therefore, treatments which encourage embryoid production do not favor normal vine development and, conversely, treatments which favor normal vine development restrict somatic embryogenesis.

We propose to use the process of secondary embryogenesis in grape for vine production as illustrated in the schematic below.



The production of vines by this process can be divided into three phases: 1) embryoid replication, 2) vine formation and 3) storage.

Each embryoid is capable of producing approximately 25 embryoids at the transition zone between the root and shoot when grown on Murashige-Skoog medium supplemented with sucrose and vitamins but without growth

regulators. The generation time is approximately 15 to 35 days at temperatures of 21<sup>o</sup> to 27<sup>o</sup>C. Our clone has been producing embryoids in this manner for more than three years.

Embryoids could be stored at 4<sup>o</sup>C or at room temperature on sucrose or nutrient deficient medium and then moved to replicative conditions or vine forming conditions. This aspect of the production scheme has not been fully explored. Vines can be placed in cold storage (after proper acclimation) and then planted in the field or they can be directly transplanted to field conditions after 2 to 3 weeks acclimation to ambient conditions.

Our initial efforts have been directed at examination of the physical and chemical factors which influence embryoid replication and development of normal vines from somatic embryoids. The parameters for embryoid initiation were % embryogenesis (the percentage of embryoids which produce embryoids) and the number of embryoids produced per embryoid. The parameters for embryoid development were extension or expansion of root, hypocotyl, or cotyledons. We also noted greening of hypocotyls, cotyledons and separation of cotyledons. Complete vine formation from embryoids occurred with a frequency of approximately 5%. We have not yet been able to experimentally alter this frequency.

Effect of Physical Factors: The frequency of embryogenesis in control cultures was approximately 60%. The frequency was enhanced by growth on liquid rotating media and removal of embryoids from the mother plant when they were 6 to 10 mm long (Table 1). The frequency was zero when embryoids were grown at 12<sup>o</sup>C or lower, or when they were vernalized at 4<sup>o</sup>C for 30 to 60 days and then returned to ambient conditions. Embryoids gradually chilled to 4<sup>o</sup>C and held at that temperature for 30 to 60 days displayed a normal frequency of embryogenesis. The transfer of embryoids when less than 5 mm long and surgical removal of the shoot and root apex of the mother plant also reduced the frequency of embryogenesis.

The number of embryoids produced per mother plant was enhanced when they were grown under 16 hour day length and by transfer of embryoids when they were 11 to 15 mm long; the number was reduced by vernalization at 4<sup>o</sup>C for 30 to 60 days, growth of mother plants in stationary liquid medium, and surgical removal of the cotyledons and shoot apex of the mother plant.

The development of mother plants and their secondary embryoids was modified by variations in the physical environment. Mother plants grown under ambient conditions elongate to 20 to 30 mm in 35 days. They have pale green hypocotyls, and white, fused, unexpanded cotyledons. The tap root reaches a length of 25 to 40 mm during this time. Laterals from the transition zone or from the tap root frequently appear. Growth at 4<sup>o</sup>C for 30 or 60 days followed by growth at room temperature promoted greening of the hypocotyl and greening, separation, and expansion of the cotyledons. In contrast, growth of plants at 35<sup>o</sup>C resulted in degreening and swelling of the hypocotyl accompanied by callus formation. Roots were short, highly branched and ageotropic. Marked differences in mother plant and

embryoid development were noted when both were grown in a liquid medium which was either rotated or stationary. In a stationary medium the root system failed to develop, whereas the cotyledons often expanded and separated. In contrast, rotating liquid or filter paper wick systems promoted rapid root elongation (50 mm or longer) and hypocotyl extension (rotating liquid) but not cotyledon expansion. Embryoids under 5 mm removed from the mother plant and grown in the light failed to elongate and often developed swollen callus-like masses. In contrast, similar embryoids grown in the dark displayed slightly more radicle and hypocotyl extension. Growth in dark promoted hypocotyl extension of larger size embryoids, and growth under high light intensities repressed root extension.

Effects of Chemical Factors: The frequency of embryogenesis was enhanced by growth of mother plants on 1/2 strength Murashige-Skoog salts (other components unchanged) or by growth on media supplemented with 0.01 to 0.1% activated charcoal. Supplementing the media with growth regulators, indole-3-acetic acid (IAA), benzyladenine (BA) gibberellic acid (GA), abscisic acid (ABA) or the ethylene-producing substance ethephon, reduced the frequency of embryogenesis depending on concentration applied. Ethanol when added after autoclaving was strongly inhibitory but had no effect if added prior to autoclaving. Mother plants grown on sucrose concentrations of 0 to 0.8% displayed low frequency embryogenesis. Mother plants transferred from 0 to 1% sucrose did not regain the capacity to form embryoids.

The number of embryoids per mother plant was increased when they were grown on 1/2 strength Murashige-Skoog salts or when the sucrose concentration was 5 to 15%. The embryoids formed under the highest concentration of sucrose were extremely small and often too numerous to count. The number of embryoids per mother plant was reduced when the medium was supplemented with any of the growth regulators, ethanol (added after autoclaving) and all concentrations of activated charcoal.

The development of embryoids, especially root and hypocotyl extension, was strongly repressed by high sucrose concentrations. BA treatment enhanced cotyledon expansion, separation and greening. Gibberellin treatment enhanced greening of hypocotyls and cotyledons and increased hypocotyl elongation. Transfer of embryoids from 0 to 1% sucrose resulted in greening of hypocotyls and cotyledons and separation of cotyledons. Ethephon treatment promoted hypocotyl expansion and increased lateral root formation but repressed cotyledon development and greening of shoots. High concentrations of IAA, BA or ethephon resulted in callus formation.

Theoretical Yield: One embryoid can produce 25 embryoids in 35 days or  $9 \times 10^{13}$  embryoids per year if each produced 25. This is a conservative estimate since embryoid production per mother plant can easily be tripled.

Genetic stability: Vines produced by somatic embryogenesis are not identical to the parent material. They are more vigorous, have red

coloration in the stems (parents are green), display a different fruit cluster shape and have a more spicy juice. Cuttings from regenerated plants root with higher frequency, have more uniform shoot growth and break dormancy earlier than cuttings from parent plants. Regenerated plants more closely resemble the description of the original hybrid than do the parent plants. The increased vigor and altered morphological characteristics of regenerated plants may be due to: elimination of virus, juvenile characteristics of regenerated plants, or altered genotype. It is also possible that current stocks of 'Seyval' are the result of a superficial chimera mutation and regeneration of cells below the chimera might have resulted in recovery of the original phenotype.

Discussion: The propagation of vines by shoot tip culture or its modifications is at present the most efficient of the cell culture methods. Production of vines via primary somatic embryogenesis (from callus) or by secondary embryogenesis (from embryoids regenerated from callus) has not yet been developed to the state of commercial exploitation. The major barrier to vine production by primary embryogenesis is a lack of knowledge on how to obtain embryogenically competent callus of cultivars other than 'Seyval' or 'Cabernet Sauvignon'.

The production of vines via secondary embryogenesis has promise of commercial exploitation for the cultivar 'Seyval'. Propagation of other cultivars by secondary embryogenesis cannot proceed until problems of primary embryogenesis have been solved. Production of large numbers of 'Seyval' embryos via secondary embryogenesis is now possible. We have obtained multiplication factors of 1 embryoid giving rise to more than 100 embryoids in 35 days. The major problem with this process is now to obtain complete development of secondary embryoids. Our data suggest that regenerated embryoids may require an after ripening period for normal development, as do sexual embryos of *Vitis*. However, the process of chilling embryoids has not alleviated all dormancies and in no case have we been able to significantly promote normal shoot apex development. We have identified several single physical and chemical factors which reduce dormancy of roots, cotyledons, or hypocotyls. The next phase of the research will involve combinations of these factors and determination of the order in which they are applied for production of normal plants.

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Table 1. Physical factors influencing embryoid initiation and development.

| Experimental variable      | % Embryo-genesis <sup>Z</sup> | Embryoids/culture <sup>Z</sup> | Embryoid development <sup>Z,Y</sup> |
|----------------------------|-------------------------------|--------------------------------|-------------------------------------|
| TEMPERATURE                |                               |                                |                                     |
| Preculture 4°C             |                               |                                |                                     |
| Temperature reduced 2°/day | 0                             | -                              | +R,+C(green)                        |
| Placed directly in 4°C     | --                            | --                             | +R,+C(green)                        |
| Culture 12°C or less       | 0                             | 0                              | 0                                   |
| Culture 35°C               | 0                             | 0                              | +R,H (callus swelling)              |
| PHYSICAL STATE OF MEDIA    |                               |                                |                                     |
| Liquid stationary          | 0                             | -                              | --R, +C                             |
| Liquid rotating            | +                             | 0                              | +R, +H                              |
| Filter paper               | 0                             | 0                              | +R                                  |
| LIGHT INTENSITY            |                               |                                |                                     |
| High                       | 0                             | 0                              | -R                                  |
| Dark                       | 0                             | 0                              | +H                                  |
| LIGHT DURATION             |                               |                                |                                     |
| Long day                   | 0                             | +                              | 0                                   |
| MEDIUM AGE                 |                               |                                |                                     |
| 2 weeks                    | 0                             | 0                              | 0                                   |
| TIME OF TRANSFER           |                               |                                |                                     |
| 4 WEEKS                    | 0                             | -                              | (callus, browning)                  |
| MEDIUM VOLUME              |                               |                                |                                     |
|                            | 0                             | 0                              | 0                                   |
| EMBRYO SIZE                |                               |                                |                                     |
| 1-5mm (light)              | -                             | 0                              | -R,-H(callus,swelling)              |
| 1-5mm (dark)               | -                             | 0                              | +R, +H                              |
| 6-10mm                     | +                             | 0                              | 0                                   |
| 11-15mm                    | 0                             | +                              | 0                                   |
| APEX REMOVAL               |                               |                                |                                     |
|                            | 0                             | -                              | 0                                   |
| ROOT REMOVAL               |                               |                                |                                     |
|                            | 0                             | 0                              | 0                                   |
| APEX + ROOT REMOVAL        |                               |                                |                                     |
|                            | -                             | 0                              | 0                                   |

<sup>Z</sup> + = promoted, - = retards, 0 = no effect.

<sup>Y</sup> R = root, C = cotyledons, H = hypocotyl.

Table 2. Chemical factors influencing embryoid initiation and development.

| Experimental variable   | % Embryo-genesis <sup>Z</sup> | Embryoids/culture <sup>Z</sup> | Embryoid development <sup>Z,Y</sup> |
|---|-------------------------------|--------------------------------|-------------------------------------|
| GROWTH REGULATORS   |                               |                                |                                     |
| IAA   |                               |                                |                                     |
| 10 <sup>-9</sup> -10 <sup>-6</sup> M                              | -                             | 0                              | 0                                   |
| 10 <sup>-5</sup> -10 <sup>-4</sup> M                              | --                            | --                             | +R, Callus                          |
| BA  |                               |                                |                                     |
| 10 <sup>-9</sup> -10 <sup>-6</sup> M                              | 0                             | 0                              | 0                                   |
| 10 <sup>-5</sup> -10 <sup>-4</sup> M                              | --                            | --                             | ++C (green callus)                  |
| GA  |                               |                                |                                     |
| 10 <sup>-9</sup> -10 <sup>-6</sup> M                              | 0                             | 0                              | 0                                   |
| 10 <sup>-5</sup> -10 <sup>-4</sup> M                              | 0                             | -                              | +H, +C (green)                      |
| ABA   |                               |                                |                                     |
| 10 <sup>-9</sup> -10 <sup>-6</sup> M                              | 0                             | 0                              | 0                                   |
| 10 <sup>-5</sup> -10 <sup>-4</sup> M                              | -                             | --                             | 0                                   |
| ETHEPHON  |                               |                                |                                     |
| 10 <sup>-9</sup> -10 <sup>-6</sup> M                              | 0                             | 0                              | 0                                   |
| 10 <sup>-5</sup> -10 <sup>-4</sup> M                              | -                             | 0                              | -C, +R, +H (callus)                 |
| NUTRIENT SALTS CONC.  |                               |                                |                                     |
| 1/2 x   | +                             | +                              | 0                                   |
| 0 x   | -                             | --                             | +R                                  |
| SUCROSE   |                               |                                |                                     |
| 0   | --                            | --                             | 0                                   |
| 1%  | -                             | 0                              | 0                                   |
| 5%  | 0                             | +                              | 0                                   |
| 10-15%  | 0                             | ++                             | --R, -H                             |
| 0 1%  | --                            | --                             | +C (green)                          |
| ORGANIC CONSTITUTENTS   |                               |                                |                                     |
| (thiamine, pyrodoxine,<br>nicotinic acid, glycine,<br>m-inositol) |                               |                                |                                     |
| 0   | (-)                           | 0                              | 0                                   |
| SOLVENTS  |                               |                                |                                     |
| Ethanol (autoclaved)  | 0                             | 0                              | 0                                   |
| Ethanol (not autoclaved)  |                               |                                |                                     |
| 0.001%-0.1%   | -                             | -                              | 0                                   |
| 1.0%  | --                            | --                             | 0                                   |
| DMF (autoclaved)  | 0                             | 0                              | 0                                   |
| ACTIVATED CHARCOAL  |                               |                                |                                     |
| 0.01%   | +                             | -                              | 0                                   |
| 0.1%  | +                             | -                              | +R, +C                              |
| 1.0%  | 0                             | -                              | ++R, +C                             |

<sup>Z</sup> + = promoted, - = retards, 0 = no effect.

<sup>Y</sup> R = root, C = cotyledons, H = hypocotyl.

## Blueberry Micropropagation

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The introduction of a new blueberry cultivar takes at least 15 years from the time the seedling is first produced. About two-thirds of this period results from the time required to propagate sufficient plants for testing of the selection and for production of plants for release to growers. Thus a rapid propagation technique becomes highly desirable, particularly when the number of stock plants available is limited. Our work on blueberry micropropagation was started at the request of the blueberry breeder in the Fruit Laboratory for the reasons outlined above.

We began our work in 1977 using Anderson's rhododendron medium (1) but this proved to be not entirely satisfactory. The following year we modified the revised medium that Anderson had developed (2) and obtained better results with this modified medium. The composition of this medium is given in Table 1. The modified medium contains much less sodium than Anderson's revised medium and only a trace of chloride. The micronutrients are those of Murashige and Skoog (MS) (4), except for concentrations of  $\text{FeSO}_4$  and  $\text{Na}_2\text{EDTA}$  which are double those of MS; the macronutrients are roughly equivalent to 1/4 strength of the MS medium.

Preparation of shoots for culture requires the use of two additional solutions. For surface sterilization of the tissues, a saturated solution of calcium hypochlorite is prepared by adding 60 grams of technical grade chemical to 1 liter of distilled water and stirring for 15 minutes. The residue is allowed to settle and the solution is filtered using a mild vacuum. From this stock, a half-strength solution containing 0.01% Tween 20 is prepared. In addition, an antioxidant solution is prepared by dissolving 75 mg of citric acid and 50 mg of ascorbic acid in 1 liter of water and autoclaving or filter sterilizing the solution.

Cultures are established from actively growing shoot tips, preferably using the first flush of growth. Shoots are cut in the greenhouse or field and dropped into distilled water for transport to the laboratory. To prepare the shoot tips, the leaves and petioles are snapped off as close to the stem as possible without damaging axillary buds. As many as possible of the tightly furled leaves around the shoot apex are loosened and removed. The shoot tips are dropped into a jar of distilled water, transferred to a laminar flow hood and one drop of Micro detergent is added for each 50 ml of water. The jar is agitated gently for 1 minute and the water is poured off. Freshly prepared calcium hypochlorite is poured over the shoot tips, the jar is agitated gently for 10 minutes and the liquid is poured off. This is followed by a sterile distilled water wash for a few seconds. The shoot tips are rinsed for a few seconds in

sterile citric acid-ascorbic acid solution, then rinsed in two changes of sterile distilled water, first for a few seconds and then for 5 minutes.

For explanting, the shoot tips are trimmed to a length of 1 to 2 cm and placed horizontally, partially embedding the full length of the shoot tip in the medium. To explant axillary buds, the shoot segments are trimmed to about 5 mm on either side of the bud. The stem is embedded in the medium so that the bud is just at the surface of the medium. Cultures are grown at 24° to 26°C with 16-hour photoperiods at a light intensity of 2,000 to 4,000 lux from warm white fluorescent lights.

Within a week, the tightly furled leaves enclosing the shoot tip starts to elongate and unfurl. As these leaves develop, they touch the surface of the medium. Adventitious buds and/or callus then develop on the leaf surface where it touches the medium. Later, adventitious buds may develop from the callus as well. We have noted adventitious bud formation on explants obtained from mature plants of highbush blueberry (*Vaccinium corymbosum* L.) and of various interspecific hybrids having two or more of the following in their parentage: *V. corymbosum*, *V. ashei* Reade (rabbiteye), *V. atrococcum* (A. Gray) A. Heller, *V. constablaei* A. Gray, *V. Darrowi* Camp, and *V. Elliottii* Chapman. Adventitious shoot formation has been observed elsewhere on lowbush (*V. angustifolium* Ait.) (5), rabbiteye (3) and highbush blueberry (D. Cohen, personal communication).

Subcultured shoots also produce adventitious buds on basal leaves which touch the medium. These may take the form of adventitious shoots growing directly from the leaf surface or of callus masses on the leaf surface which in turn produce adventitious buds and/or shoots. Sometimes the buds themselves produce further adventitious buds before they have elongated.

In addition to adventitious buds, axillary buds on the original explant, and on shoots produced in culture, grow and develop. When a mass of shoots develops on an explant, the origin (axillary or adventitious) can be determined only by dissecting and/or sectioning the tissue.

With the relatively high level of cytokinin (15 mg/liter of 6- $\gamma$ ,  $\gamma$ -dimethylallylamino purine) (2iP) used, both axillary and adventitious shoots elongate very little and have extremely short internodes. To test the effect of cytokinin concentration on shoot growth, we subcultured shoot explants on media containing 0, 3.75, 7.5, 15 or 30 mg/liter of 2iP. The numbers of new shoots and buds produced were directly related, and shoot length was inversely related, to the 2iP concentration. Varying the concentration of indoleacetic acid (IAA) from 0 to 8 mg/liter in the same experiments had little effect on shoot proliferation or elongation, although there was some tendency for higher IAA concentrations to slightly depress the number of new shoots produced.

Transferring clumps of shoots from a medium with a high level of cytokinin (15 mg/liter 2iP) to one with a low level (1 to 5 mg/liter 2iP) or none permits the existing shoots to elongate sufficiently so that they can be easily collected for rooting.

Early attempts to root cuttings on an agar medium had only limited success. Even when rooting percentages were reasonably high, large masses of callus formed and the adventitious roots grew in the air rather than in the medium. This occurred whether or not auxin was present in the medium.

As a result, we decided to attempt rooting the cuttings by conventional methods. Shoots were collected under sterile conditions so that the remainder of the culture could be transferred to fresh medium. The shoots were accumulated in a jar of water until all had been collected. Cuttings were prepared by trimming shoots to a length of 2 to 3 cm, dipping the basal end in 0.1% IBA on talc, and inserting the cuttings into milled sphagnum in plastic flats. The flats were placed under intermittent mist in the greenhouse. Adventitious root initiation began within 3 weeks and cuttings developed a mass of fibrous roots by 5 weeks. Rooting varied among the selections with 33 to 65% of the cuttings rooted after 3 weeks increasing to 72 to 90% by 7 weeks.

Further experiments have been done using a wider range of selections and cultivars. Cuttings root as well using plastic sheeting to maintain a high humidity as they do under intermittent mist. Briefly dipping the bases of the cuttings in 1000 mg/liter of IBA in 50% ethanol has been more effective in some cases than the IBA on talc and has never given poorer results.

Rooted cuttings are easily acclimated to greenhouse conditions by gradually reducing the humidity or the amount of mist applied. Rooted cuttings are then potted in peat or a peat-soil potting mix and grown in a manner similar to that used for blueberry seedlings.

Some blueberry plants from tissue culture were planted in the field in 1979 and others will be planted in 1980. Thus none of the tissue-cultured plants has flowered or fruited. Although no problems in fruiting are anticipated, it would be premature to recommend this technique for wholesale propagation of blueberries for commercial production. However, we think that the method has great potential for the future, once field testing has been satisfactorily concluded.

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Table 1. Composition of medium used for blueberry tissue culture.

| Component  | Amount per liter |        |
|--|------------------|--------|
|  | mmol             | mg     |
| NH <sub>4</sub> NO <sub>3</sub>                      | 2                | 160    |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>      | 1.5              | 198    |
| Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O | 3                | 708    |
| KNO <sub>3</sub>                                     | 2                | 202    |
| KH <sub>2</sub> PO <sub>4</sub>                      | 3                | 408    |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O                 | 1.5              | 370    |
| FeSO <sub>4</sub> ·7H <sub>2</sub> O                 | 0.2              | 55.7   |
| Na <sub>2</sub> EDTA                                 | 0.2              | 74.4   |
| MnSO <sub>4</sub> ·H <sub>2</sub> O                  | 0.1              | 16.9   |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                 | 0.03             | 8.6    |
| H <sub>3</sub> BO <sub>3</sub>                       | 0.1              | 6.2    |
| KI   | 5.0 µmol         | 0.83   |
| Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O  | 1.0 µmol         | 0.25   |
| CoCl <sub>2</sub> ·6H <sub>2</sub> O                 | 0.1 µmol         | 0.025  |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                 | 0.1 µmol         | 0.025  |
| myo-inositol   | 0.56             | 100    |
| Adenine sulfate·2H <sub>2</sub> O                    | 0.20             | 80     |
| Thiamine HCl   | 1.2 µmol         | 0.4    |
| IAA  | 5.7 µmol         | 4      |
| 2iP  | 73.5 µmol        | 15     |
| Sucrose  | 87.6             | 30,000 |
| Agar   | ---              | 5,500  |

The pH is adjusted to 4.8 before adding agar.

## Peach Micropropagation

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The technique of propagating trees by grafting dates back at least to Roman times (10). Today, peach trees are propagated primarily by bud-grafting (9). This practice is time consuming, requires skilled labor, and permits only limited plant increases. However, it is carried out because, until recently, peach cuttings could not be rooted in satisfactory percentages by methods available (4, 6, 9). Micropropagation or in vitro propagation through enhancement of axillary shoot formation enables timely increase and hastens the availability of new cultivars. Even with cultivars that are propagated readily by asexual techniques, the tissue culture method can be utilized to enhance substantially the rate of multiplication. A millionfold increase per year in the rate of clonal multiplication over conventional methods is not unrealistic (13). In addition, micropropagation of virus-free stock plants promises that disease-free cultivars can be made available throughout the world.

Micropropagation of many herbaceous plants has been successfully accomplished (13). Within the last few years, commercial plant tissue culture laboratories have been designed to partially fill the plant propagation needs of the parent nursery, and a few facilities sell plants in tubes as novelty items (5). For many years, woody species were considered difficult in vitro material. One major problem was the difficulty in obtaining uncontaminated cultures from mature plants, even after disinfection (19). Recent work suggests that woody plants are amenable to culture and the potential for multiplication has now been demonstrated in at least 40 species (1).

Prunus shoot tip culture. Very little has been reported on shoot proliferation of peach cultivars (8, 19, 20). Considerably more has been reported on micropropagation of rootstocks and scions of other Prunus species (2, 11, 15, 16, 17, 18, 21, 22). Quoirin et al. (17) recently published a 10-year report on in vitro multiplication of Prunus and Malus species from meristems. They found Heller's micronutrient solution (7) with increased Mn (10x) to be superior to Murashige and Skoog's (MS) formulation (14). Later, they switched to the formulation of Lepoivre (17). Close inspection of their data reveals that microelement requirements vary from species to species. A comparison of the different micronutrient formulations is presented in Table 1. With the exception of Mn and I, the Lepoivre and MS formulations are almost identical.

A divergence of opinion exists regarding the suitability of MS macronutrients for Prunus shoot tip culture (16, 18, 19, 20, 21, 22). In my laboratory we have successfully cultured peaches on MS medium (8). Lepoivre macronutrients contain no  $\text{CaCl}_2$  and one-fourth of the amount of  $\text{NH}_4\text{NO}_3$  present in MS. A comparison of nitrogen sources in different

macronutrient formulations (Table 3) used for *Prunus* tissue culture reveals that MS, Lepoivre, and Miller (12) macronutrients have high levels of total N; however, the proportion of  $\text{NO}_3\text{-N}$  to  $\text{NH}_4\text{-N}$  differs. Modified Knops (18) has a low level of  $\text{NO}_3\text{-N}$  and no  $\text{NH}_4\text{-N}$ .

Quoirin et al. (17) and Zuccherelli (22) claim that a period of elongation following multiplication predisposes the shoots to root in response to auxin. Pretreatment for a month at 4°C on multiplication medium containing gibberellic acid ( $\text{GA}_3$ ) stimulates elongation. The addition of phloroglucinol did not stimulate additional rooting. Rosati et al. (18) demonstrated a definite interaction between the addition of  $\text{GA}_3$  to the rooting medium, growth room temperature and percent rooting. The inhibition of rooting which occurred at 26-30°C at all concentrations of indole-3-butyric acid (IBA) tested was overcome by  $\text{GA}_3$  but had no effect at 15°C or 21°C.

Peach shoot tip culture. Attempts to duplicate the work of Skirvin (19, 20) were unsuccessful and we set out to determine optimum conditions for Stage I (explant establishment), Stage II (multiplication), and Stage III (rooting) (13) of a wide variety of peach scion cultivars and peach and plum rootstocks. Thus far, we have worked with scion cultivars 'Compact Redhaven', 'Redhaven' and 'Sunhigh', peach rootstocks 'Lovell', 'Halford', and 'Boone County' and plum rootstock Myrobalan.

The major problems experienced in Stage I were contamination and unsuitability of the media. These problems have also been reported by others working with *Prunus* (2, 19, 21). We found that regardless of the type of surface sterilant used, it was impossible to successfully culture dormant buds. We found that contamination can be reduced from 100% to 3.6% by the following technique. Buds are forced, leaves removed, and the shoots trimmed to 5 mm and then surface sterilized with 0.5% sodium hypochlorite and 0.01% Tween 20 for 15 min, and 100-500 ppm pen-strep (a commercial preparation of penicillin and streptomycin) for 15 min.

Optimum growth and minimum necrosis and callus formation occurred on MS media supplemented with (in mg/liter) 0.1 p-aminobenzoic acid, 0.5 pyridoxine, 0.5 nicotinic acid, 0.4 thiamine, 100 inositol, 0.2 benzyladenine (BA), and 0.01 to 0.1 IBA. Substitution of kinetin for BA or  $\alpha$ -naphthaleneacetic acid for IBA resulted in poor growth or death of the tissue. Benzyladenine at 1.0 to 5.0 mg/liter was inhibitory. Growth on liquid media was significantly greater than growth on solid media. Some cultivars grew best on filter wicks in vials while others grew best in test tubes on a roller drum.

In general, shoot tips from rootstock cultivars multiplied better than those from scion cultivars. A minimum of 4 weeks at Stage I was required for successful Stage II. Shoot tips multiplied best on Stage I medium in which BA was increased to 1.0 mg/liter from 0.2 mg/liter. In 7 to 8 weeks, a proliferation rate of 10:1 and 5:1 was possible for shoot tips from rootstock and scion cultivars, respectively. Experiments are under way to determine if a maximum proliferation rate can be achieved in a shorter period of time.

To date, there are no reports in the literature on successfully rooting peach shoots cultured in vitro. Experiments have been designed to determine optimum conditions for rooting. Our studies include the effects of different mineral salts, auxins, temperature, and GA<sub>3</sub> on rooting. In the future, studies will be conducted to compare vigor, tree size, and phenotypic stability of scion cultivars on their own roots with trees grafted on normally propagated rootstocks. Thus far, other Prunus species propagated by axillary shoot enhancement show no genetic aberrations or abnormalities (3).

Micropropagation represents a new approach to peach propagation. It has the potential of providing the propagator with many identical plants which are relatively free of pathogens. The procedure can be initiated at any time of the year, and can be accomplished in a fairly short time and within a limited space. These facts should be extremely useful to those interested in rapid and massive propagation of new cultivars or to meet a particularly high demand of the market.

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Table 1. Comparison between the different formulation of microelements.

| Microelements <sup>z</sup>                          | Heller plus<br>Mn 10x (7) | Murashige and<br>Skoog (14) | Lepoivre<br>(17) |
|---|---------------------------|-----------------------------|------------------|
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                | 1.0                       | 8.6                         | 8.6              |
| H <sub>3</sub> BO <sub>3</sub>                      | 1.0                       | 6.2                         | 6.2              |
| MnSO <sub>4</sub> ·4H <sub>2</sub> O                | 1.0                       | 22.3                        | 1.0              |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                | 0.03                      | 0.025                       | 0.025            |
| NiCl <sub>2</sub> ·6H <sub>2</sub> O                | 0.03                      | -                           | -                |
| AlCl <sub>3</sub>                                   | 0.03                      | -                           | -                |
| KI  | 0.01                      | 0.83                        | 0.080            |
| Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O | -                         | 0.25                        | 0.25             |
| CoCl <sub>2</sub> ·6H <sub>2</sub> O                | -                         | 0.025                       | 0.025            |

<sup>z</sup> Microelements in mg/liter.

Table 2. Comparison between different formulations of macroelements.

| Macroelement <sup>z</sup>                            | Murashige and<br>Skoog (14) | Lepoivre (17) |
|--|-----------------------------|---------------|
| NH <sub>4</sub> NO <sub>3</sub>                      | 1650                        | 400           |
| KNO <sub>3</sub>                                     | 1900                        | 1800          |
| CaCl <sub>2</sub> ·2H <sub>2</sub> O                 | 440                         | -             |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O                 | 370                         | 360           |
| KH <sub>2</sub> PO <sub>4</sub>                      | 170                         | 270           |
| Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O | -                           | 1200          |

<sup>z</sup> Macroelements in mg/liter.

Table 3. Comparison of nitrogen source in different nutrient media.

| Media                    | Concentration (g/liter) |                    |                    |
|--------------------------|-------------------------|--------------------|--------------------|
|                          | Total N                 | NH <sub>4</sub> -N | NO <sub>3</sub> -N |
| Murashige and Skoog (14) | 5.20                    | 1.65               | 3.55               |
| Modified Knops (21)      | 1.34                    | -                  | 1.34               |
| Lepoivre (17)            | 5.00                    | 0.40               | 4.60               |
| Miller (12)              | 3.50                    | 1.00               | 1.50               |

## Micropropagation of Fruit Tree Rootstocks

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Rootstocks of apple (Antonovka KA313, EMLA-7, EMLA-9, EMLA-27, MAC-9), cherry (Colt, Mahaleb x Mazzard 14), pear (Old Home x Farmingdale 51), and plum (Pixy, St. Julien X) were successfully produced using tissue culture techniques. Cultures were initiated from actively growing shoot tips. The medium used for all stages contained the macronutrient elements of Murashige and Skoog (MS) at half strength, MS micronutrient elements with some modifications, 250 mg/liter myo-inositol, 2.5 mg/liter thiamine HCl, 30 g/liter sucrose and 6 g/liter agar. For shoot proliferation, 5  $\mu$ M (approx. 1 mg/liter) benzyladenine was used in combination with 0.5 - 5.0  $\mu$ M (approx. 0.1 - 1.0 mg/liter) indolebutyric acid (IBA) or 0.5 - 50 nM (approx. 0.1 - 10.0  $\mu$ g/liter) naphthaleneacetic acid or 0.5 - 50 nM (approx. 0.1 - 10.0  $\mu$ g/liter) 2,4-dichlorophenoxyacetic acid. For rooting, only IBA at concentrations up to 5  $\mu$ M (approx 1 mg/liter) were used. Additional details on the techniques used are available in the following publications:

Cheng, T.-Y. 1978. Clonal propagation of woody plant species through tissue culture techniques. Proc. Int. Plant Prop. Soc. 28:139-155.

Cheng, T.-Y. 1979. Micropropagation of clonal fruit tree rootstocks. Compact Fruit Tree 12:127-137.

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<sup>1</sup> Dr. Cheng was unable to submit a manuscript covering the material she presented in her talk.

## Apple Cultivar Micropropagation

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Apple cultivars are propagated by budding or grafting on seedling or vegetatively propagated rootstocks. The potential for growing apple cultivars on their own roots under modern orchard cultural practices has not been evaluated because of the difficulty in propagating own-rooted trees. Tissue culture offers the means of developing a useful rapid propagation technique for apples. Part of the work reported here has been briefly described previously (10).

Progress on tissue culture propagation of apples was reported in 1976 by Abbott and Whiteley (1) and Jones (3). The Jones method for M.26 apple rootstock (3, 4) involved the use of phloridzin or phloroglucinol added to the Linsmaier and Skoog (7) modification of the Murashige and Skoog (MS) high-salt medium (8). More recently, he and his coworkers extended this technique to several apple cultivars (5). Huth (2) and Lane (6) reported successful propagation of 'Jonathan' and 'McIntosh' apple, respectively.

We use two methods for establishing cultures of apple cultivars. Most often, we collect actively growing shoot tips, 7 to 10 cm (3 to 4 in.) long, in the orchard or greenhouse. The leaves are removed, taking care to remove the stipules but not to damage the stem around the lateral buds. The shoot tips are dropped into distilled water and 2 drops of Micro detergent are added per 100 ml of water. The shoots are gently agitated for 5 minutes and then the water is poured off. To further cleanse the shoots, they are rinsed 2 or 3 times in distilled water. Half-strength calcium hypochlorite (60 g/liter, stirred 15 minutes, then filtered under vacuum and prepared fresh daily) plus 0.01% Tween 20 is added and the shoots are stirred gently for 20 minutes using a magnetic stirrer. The shoot tips are rinsed twice in sterile distilled water, first for a few seconds and then for 5 minutes with constant stirring.

The shoot tips are prepared for explanting by trimming them to 10 to 15 mm (1/2 in.) in length and placing them in 15 ml of MS medium (Table 1) in 125 ml Erlenmeyer flasks sealed with aluminum foil. The flasks are rotated continuously at 1 rpm for 2 to 4 days. Some tissue damage occurs during the tissue disinfection procedure, with the result that phenolic compounds leak from the tissue into the culture medium. When the shoot tips are first cultured in liquid medium and then transferred to solid medium, growth is superior and losses are lower than when the shoot tips are placed directly onto solid medium.

When the shoot tips are transferred to solid medium, they are positioned horizontally on the medium and embedded for half their thickness. Growth of the terminal and lateral buds usually starts within

7 to 10 days. After 3 weeks, the larger leaves are trimmed off and the shoot tips are transferred to fresh medium. Transfers are then made every 3 to 4 weeks, dividing the clumps of shoots as necessary. Cultures are grown at 24° to 26°C with 16-hour photoperiods at a light intensity of 2,000 to 4,000 lux from warm white fluorescent lights.

The other method for shoot establishment is to dissect meristem-tips about 0.5 mm high from dormant buds under aseptic conditions. Dormant shoots are collected in the field after the rest period has been satisfied; the shoots are used immediately or stored at 2°C until needed. Shoots are cut into sections about 2 to 4 cm long with a bud close to the upper end of the section. Doing so facilitates handling the stem away from the bud during dissection. Shoot segments are agitated in 95% ethanol containing 1 drop of Tween 20 per 100 ml for 10 minutes and then in full strength calcium hypochlorite solution for 20 minutes. The segments are rinsed in sterile distilled water and held in sterile distilled water until the buds are dissected.

The buds are dissected by sequentially removing the bud scales and then the leaf primordia to expose the growing point. The sterile forceps and scalpels used for dissecting the meristem-tip are changed several times during the procedure to reduce the chance of contaminating the tip. The growing point is excised together with one or two leaf primordia and explanted with the basal end resting on the surface of the medium. To initiate the culture, we use 10 ml of a modified Lepoivre's medium (9) (Table 1) in a scintillation vial. Care must be taken during dissection and explanting to prevent the meristem tip from desiccating. Therefore, this step is not done in a laminar flow hood but in a quiet corner of the laboratory free of drafts.

Cultures are grown for 6 to 8 weeks in the vials. The growing tips are then transferred to proliferation medium and handled in the same way as cultures established from actively growing shoot tips.

Following Jones' technique, we included phloroglucinol in the medium. Since we could find no consistent benefit from using phloroglucinol for shoot proliferation, it was eliminated from the medium.

When sufficient shoots become available, they can be harvested for rooting and the remaining stem pieces recultured to produce more shoots. Cuttings are prepared by trimming the shoots to 2 to 4 cm long.

Three methods of rooting cuttings have been tried: (a) rooting in aseptic conditions on agar medium; (b) rooting in aseptic conditions in liquid medium with support for the cuttings provided by vermiculite, perlite, or sand; and (c) rooting in non-aseptic conditions under a plastic tent or under intermittent mist.

For rooting in aseptic conditions, we use half strength MS, without benzyladenine (BA) and gibberellic acid (GA<sub>3</sub>), and adjusting the concentration of indolebutyric acid (IBA). For rooting in agar medium, 0.1 to 0.3 mg/liter of IBA have given the best results for the cultivars we have tested. In liquid medium with vermiculite, perlite, or sand as the support for cuttings, IBA could be reduced to 0.01 mg/liter, or be completely eliminated, and rooting percentages were as good as, or better than, with higher IBA concentrations.

Several factors affecting rooting of apple cultivar cuttings under aseptic conditions have been studied. Addition of phloroglucinol to agar-solidified medium gave no consistent results for eight cultivars ('Delicious', 'Northern Spy', 'Nugget', 'Ozark Gold', 'Spartan', 'Spuree Rome', 'Stayman', and 'Summer Rambo') tested. Only with 'Spartan' did phloroglucinol significantly increase rooting above the controls. However, 'Spartan' cuttings rooted as well on liquid medium, having vermiculite or 1:1 vermiculite-perlite as a support phase, without phloroglucinol and auxin as on agar medium with phloroglucinol and auxin. The rooting of 'Spartan', 'Stayman', and 'Summer Rambo' was not influenced whether the phloroglucinol used in the medium was filter-sterilized or autoclaved. Varying phloroglucinol from 18 to 486 mg/liter had no effect on rooting of 'Northern Spy' cuttings.

Naphthaleneacetic acid (NAA) at 0.03 to 0.3 mg/liter stimulated more rapid rooting of 'Summer Rambo' and 'Ozark Gold', but not of 'Rome Beauty', than did equal concentrations of IBA. By the end of 4 weeks, the differences in rooting resulting from the different auxins had disappeared. Varying the agar concentration from 4.5 to 8.5 g/liter and the sucrose concentration from 15 to 60 g/liter had no effect on rooting of cuttings for the several cultivars tested. Eliminating all sucrose from the rooting medium reduced rooting to about one-quarter of that obtained with any of the sucrose concentrations tested.

Rooting of apple cuttings directly under intermittent mist or in polyethylene tents would have several advantages. Aseptic techniques would not have to be used in handling the cuttings once they are removed from the mother culture and acclimation to greenhouse conditions could be done at the same time cuttings are rooting. We have tested this method on limited numbers of cuttings for several cultivars. Rooting percentages have varied from 0 to 100%, depending upon the cultivar and treatment conditions. The results have indicated that a well-drained rooting medium is essential. The amount of intermittent mist applied must be reduced to the minimum necessary to prevent the leaves from desiccating. Applying mist for 1 second every 3 to 6 minutes is adequate in most cases, and the mist may need to be applied 24 hr per day. It may be necessary to add water occasionally to the rooting medium to maintain an adequate moisture level for cuttings being rooted under mist or under plastic.

The most difficult procedure for us has been to acclimate rooted apple cuttings to greenhouse conditions. Leaves produced in vitro seem to lack or to have an incomplete cuticle so that they dry very readily. The problem is to provide enough water or humidity to keep the leaves from

desiccating without damaging the roots from overwatering. New leaves produced under greenhouse conditions seem to have an intact cuticle. Once new growth starts under mist or plastic, the rooted cuttings begin to grow well. Some of the leaves present or formed in vitro soon die and drop off but the plants grow quite rapidly. By this time, the plants are fully acclimated to greenhouse conditions. They can then be transplanted to a nursery or the field when they are 15 to 25 cm (6 to 10 in.) tall.

We established an orchard of own-rooted apple trees in 1979 containing 11 to 62 trees each of 'Golden Delicious', 'Northern Spy', 'Ozark Gold', 'Spartan', 'Stayman', 'Summer Rambo', and 'York Imperial'. Additional cultivars have already been propagated for field planting in 1980 and plans for replicated field trials comparing own-rooted trees with ones propagated on several different rootstocks are under way.

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Table 1. Composition of media used for establishment, proliferation and rooting of apple cultivars.

| Component  | Murashige and Skoog medium <sup>Z</sup><br>(Shoot tip establishment<br>proliferation and rooting) |          | Lepoivre medium <sup>Y</sup><br>(Meristem<br>establishment) |          |
|--|---|----------|---|----------|
|  | mM  | mg/liter | mM  | mg/liter |
| NH <sub>4</sub> NO <sub>3</sub>                      | 20.6  | 1650     | 5.0   | 400      |
| Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O | --  | --       | 5.1   | 1200     |
| KNO <sub>3</sub>                                     | 18.8  | 1900     | 17.8  | 1800     |
| CaCl <sub>2</sub> ·2H <sub>2</sub> O                 | 3.0   | 440      | --  | --       |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O                 | 1.5   | 370      | 1.5   | 360      |
| KH <sub>2</sub> PO <sub>4</sub>                      | 1.2   | 170      | 2.0   | 270      |
| FeSO <sub>4</sub> ·7H <sub>2</sub> O                 | 0.1   | 27.8     | 0.1   | 27.8     |
| Na <sub>2</sub> EDTA                                 | 0.1   | 37.2     | 0.1   | 37.2     |
| MnSO <sub>4</sub> ·H <sub>2</sub> O                  | 0.1   | 16.9     | 0.1   | 16.9     |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                 | 0.03  | 8.6      | 0.03  | 8.6      |
| H <sub>3</sub> BO <sub>3</sub>                       | 0.1   | 6.2      | 0.1   | 6.2      |
| KI   | 5.0 $\mu$ M   | 0.83     | 5.0 $\mu$ M   | 0.83     |
| Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O  | 1.0 $\mu$ M   | 0.25     | 1.0 $\mu$ M   | 0.25     |
| CoCl <sub>2</sub> ·6H <sub>2</sub> O                 | 0.1 $\mu$ M   | 0.025    | 0.1 $\mu$ M   | 0.025    |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                 | 0.1 $\mu$ M   | 0.025    | 0.1 $\mu$ M   | 0.025    |
| myo-inositol   | 0.56  | 100      | 0.56  | 100      |
| Nicotinic acid                                       | --  | --       | 4.1 $\mu$ M   | 0.5      |
| Pyridoxine HCl                                       | --  | --       | 1.3 $\mu$ M   | 0.5      |
| Thiamine HCl   | 1.2 $\mu$ M   | 0.4      | 1.2 $\mu$ M   | 0.4      |
| Benzyladenine  | 4.44 $\mu$ M  | 1.0      | 0.44 $\mu$ M  | 0.1      |
| Indolebutyric acid                                   | 0.49 $\mu$ M  | 0.1      | 0.05 $\mu$ M  | 0.01     |
| Gibberellic acid                                     | 1.30 $\mu$ M  | 0.5      | --  | --       |
| Sucrose  | 87.6  | 30,000   | 58.4  | 20,000   |
| Agar (Phytagar)                                      | --  | 4,800    | --  | 4,800    |
| pH   | 5.2   |          | 5.0   |          |

<sup>Z</sup> Murashige and Skoog (8) mineral salts and Linsmaier and Skoog (7) vitamins, except that 16.9 mg/liter of MnSO<sub>4</sub>·H<sub>2</sub>O has been substituted for 22.3 mg/liter of MnSO<sub>4</sub>·4H<sub>2</sub>O.

<sup>Y</sup> Lepoivre medium (9) uses 4.5  $\mu$ M (1 mg/liter) MnSO<sub>4</sub>·4H<sub>2</sub>O and 0.5  $\mu$ M (0.08 mg/liter) KI. We have used the formulation shown which contains MS micronutrients.

# In Vitro Propagation of 'Seckel' Pear<sup>1</sup>

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**Abstract.** Proliferation of shoot tips of pear (*Pyrus communis* L. cv. Seckel) was obtained on Murashige and Skoog (MS) medium containing benzyladenine (BA), gibberellic acid (GA<sub>3</sub>) and naphthaleneacetic acid (NAA). Subculturing shoots on MS medium supplemented only with 2 mg/liter BA resulted in the highest rate of shoot multiplication. Rooting was achieved by transferring individual shoots to MS medium containing NAA. Plantlets were successfully transferred to soil.

Tissue culture techniques provide a means of rapid clonal propagation of plants and eliminate the expense involved in maintaining large numbers of stock plants required for conventional propagation. Many genera of herbaceous plants are being commercially propagated using such techniques (3, 8). Successful in vitro propagation of many fruit plants including apple (5, 6), plum (4, 10), cherry (4) and blackberry (1) have recently been reported. This report describes the procedure for pear propagation through tissue culture. The cultivar 'Seckel' was selected because of its resistance to fireblight.

## Materials and Methods

Shoot tips, about 5 cm in length, were excised from greenhouse grown 'Seckel' pear in June, when they had ceased active growth. Following surface sterilization in a solution containing 0.52% sodium hypochlorite (10% Clorox) and 0.1% Tween-20 for 10 min, the tips were cut to a length of about 2 cm, resterilized for 5 min and rinsed three times in sterile distilled water.

To induce shoot proliferation, the explants were cultured on 15 ml of Murashige and Skoog medium (9) supplemented with (per liter) 100 mg myo-inositol, 0.4 mg thiamine, 1 mg BA, 0.5 mg NAA and 0.1 mg GA<sub>3</sub>, or a medium of similar composition with the addition of 162 mg phloroglucinol. The media contained 30 g/liter sucrose and 7 g/liter agar. The pH of the media was adjusted to 5.7-5.8 with HCl or NaOH prior to sterilization by autoclaving for 15 min at 121°C. Phloroglucinol was added to the autoclaved medium by Millipore filtration.

The shoots produced in vitro were subcultured at 5 week intervals on the medium described above (without phloroglucinol) or utilized for other shoot proliferation or rooting experiments.

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To induce root formation, single shoots were transferred to 15 ml of MS or modified-MS medium supplemented with either indolebutyric acid (IBA) or NAA.

All cultures were maintained under 16 or 24 hr illumination provided by a 1:1 combination of daylight and Gro-lux fluorescent lights (about 3.4 klx) at 25 ± 1°C. Unless otherwise stated all experiments contained 10 replicated cultures and data on shoot proliferation and rooting were recorded after a duration of 5 and 4 weeks respectively.

## Results

Shoot Proliferation: About 30% of the shoot tip explants showed varying degrees of tissue or media discoloration and were discarded. The remaining shoot tips produced between 2 and 8 shoots after 5 weeks in culture, with an average of 5 shoots per tip. The addition of phloroglucinol did not enhance the rate of shoot proliferation and was not used subsequently. Subculturing 20 individual shoots onto fresh medium resulted in a 5-fold increase in the number of shoots produced. A mass of brownish callus developed at the base of the shoots. After harvesting the shoots, the calli with the remnants of the shoots were recultured on fresh medium and developed an average of 8 shoots per callus. These calli have been repeatedly sectioned and recultured (Fig. 1A).

A comparison of the effects of using the shoot proliferation medium (containing BA, NAA and GA) versus medium supplemented only with varying BA levels was conducted using shoots multiplied *in vitro*. BA at 2 mg/liter yielded the largest number of shoots (Table 1). Increasing BA levels, however, tended to reduce shoot length. All shoots proliferated in the 4 mg/liter BA treatment were less than 5 mm in length.

Root Initiation: Rooting was initially attempted by transferring single shoots to MS media containing 3 mg/liter IBA. After 4 weeks 3 of 20 cultures (15%) had rooted. Rooting was not increased by using either MS medium supplemented with 3 mg/liter IBA and 162 mg/liter phloroglucinol, 1/2 strength MS medium with 3 mg/liter IBA or MS medium, without potassium iodide, but containing 3 mg/liter IBA. All these treatments resulted in a large mass of callus developing around the end of the shoot embedded in the agar. Shoots placed on MS medium containing 2 mg/liter NAA developed minimal callus until root initials were formed. Although 80% rooting was obtained, the roots tended to develop callus-like tissue at the point of origin and had poor development of laterals (Fig. 1B). Lower concentrations of NAA were employed to improve root development (Table 2). At the lower NAA levels root development was greatly improved and there was little or no callusing at the point of origin.

Rooted plantlets were transferred to sterile vermiculite and covered with beakers to maintain a humid environment. Following acclimatization, plants were transferred to potting mix containing equal parts of peat and vermiculite (Fig. 1C).

## Discussion

These results show that although good shoot proliferation is achieved on MS medium containing BA, NAA and GA<sub>3</sub>, yet adding BA alone enhances the rate of shoot multiplication. This is in agreement with work done on apple (7). However, using only BA in the medium results in very small shoots, indicating that GA<sub>3</sub> may be necessary to induce shoot elongation. Phloroglucinol has been determined to be effective in shoot proliferation and growth of apple and plum shoot tips (4, 6) but at the concentration used it did not appear to elicit a similar response in pears.

Root development was enhanced at lower NAA concentrations. High levels of auxin (either IBA or NAA) resulted in callus formation at the base of the shoot and limited rooting or poor root development. Similar results have earlier been reported in Douglas fir (2).

This study shows that as with other fruit plants, shoot tips of pear can be utilized to achieve rapid plant propagation in vitro.

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Table 1. The effect of BA, NAA and GA<sub>3</sub> on shoot proliferation, of 'Seckel' pear after 8 weeks.

| Concentration (mg/liter) |     |                 | No. of new shoots<br>per culture |
|--------------------------|-----|-----------------|----------------------------------|
| BA                       | NAA | GA <sub>3</sub> |                                  |
| 1.0                      | 0.5 | 0.1             | 3.4                              |
| 1.0                      | 0   | 0               | 6.1                              |
| 2.0                      | 0   | 0               | 13.6                             |
| 4.0                      | 0   | 0               | 7.5                              |

Table 2. The effect of NAA levels on rooting of 'Seckel' pear shoots.<sup>2</sup>

| NAA (mg/liter) | % Rooting |
|----------------|-----------|
| 0              | 0         |
| 0.5            | 45        |
| 1.0            | 40        |
| 1.5            | 30        |
| 2.0            | 35        |

<sup>2</sup> Results of two separate experiments, each containing 10 shoots per treatment.



**A**



**B**



**C**

Figure 1. A: Shoot multiplication on medium containing BA + NAA + GA<sub>3</sub>.  
B: Root formation on shoots on medium containing 2 mg/liter NAA.  
C: Plantlet in soil.

## Transcription of Panel Discussion on Genetic Stability of Tissue Culture Propagated Plants

G. W. Schaeffer:

The topic for discussion is genetic stability of tissue culture propagated plants. Now, genetic stability is a little bit like the weather. Everybody talks about it but it is a little difficult to do anything about it. Nonetheless, we have a panel of experts with us today who have been working in propagation and tissue culture for a number of years. You know most of them and have heard some of them speak today. We'd like to have you in the audience have a good opportunity to interact with these individuals. Ask them questions and get their replies. What I'd like to have each of the panel members do is give a 5 to 7 minute narration of their ideas on genetic stability and the problems of genetic stability with the systems they've worked with and then open the floor to questions as we go across the whole panel.

Genetic stability, if I may make just one or two preliminary remarks to stimulate your thinking, is perhaps a little like the 3 blind men who were asked to describe the elephant. I'm not going to relate the story except one was assigned to the tail, one to the leg and the other one to the trunk and obviously their perspectives were quite different depending upon which system they were working with. On the one hand, the evolutionists might say genetic stability is a very good thing because it now opens up the possibilities for progress in evolution. On the other hand, the commercial grower who supplies a stock of a described type can't afford to have his primary standard appear to be something else than what he thought it was. Another anomalous aspect of genetic stability might be that in tissue culture, for example, there are large changes, particularly if the cells are cultured for a long time. One of the most obvious might be that there is a loss in the capacity of the cells to differentiate. We have to learn to deal with epigenetic effects, characteristics that are inherited for periods of time but are not true breeding changes. But on the other hand, even though we see large variabilities in tissue culture, if someone is looking for a mutant of a biological pathway, an auxotroph if you will, you have a very, very hard time finding it. I think that there are less than five described in the literature. The question arises why this is and so I hope that some of these items might be touched upon a little bit this afternoon. Some of the questions that might be asked: what is the role of the genotype of the cell? That is, some genotypes are more disstable than others. That is perhaps self evident. What does age have to do with genetic stability? How about the hormones? I have heard people say that they've eliminated 2,4-D and NAA from their systems. Does that impinge upon genetic stability of the system? The growth rates themselves - certain stress factors might induce certain genetic instability. What about temperature? How about exposure to virus and diseases? The chromosomal changes themselves - rearrangements, translocations, inversions - all of these things do occur in tissue culture. I think many of them are not very well described but there is quite a challenge in this area. So then without any further ado, I'd like to call upon the panel which is described for you in the program and

they are Dr. Carmine Damiano, Dr. Donald Scott, retired from the USDA, Dr. John McGrew, Dr. William Krul, and Dr. Richard Zimmerman.

Dr. Damiano, what are the primary aspects of genetic stability that you have to deal with in your business?

C. Damiano

The vegetative habit of strawberries originated in vitro (micro-propagation) looks quite different from that of runner-propagated plants. Although there is a difference in the formation of the shoots, I cannot say that it is a mutation. It is only a physiological trouble. It is the same, more or less, for the flowering. Cold-stored (frigo) runner-propagated plants flowered less than the meristem plants but also in this case I cannot say that is is a mutation. The frigo plants have well shaped fruit with good size, and so on but the fruit are not so numerous. The meristem plants, originated by in vitro culture and put directly for the production of fruit, have smaller fruit but also in this case, and I will explain after why not, I cannot say that it is a mutation or something like an instability of genetic behavior. For 'Aliso', I haven't found any difference between micropropagated plant and frigo plant and fresh traditional plant. Now I'm going to explain why I cannot say the difference is due to a mutation. The last slide shows the production per plant of micropropagated plants directly originated in in vitro culture [see Table 4 of C. Damiano, Strawberry micropropagation, p. 20 of these Proceedings]. In a poly house, production per plant was 207 grams and increases to 338 grams in the open field. The first fresh micropropagated plants, and I mean the daughter [runner] of this micropropagated plant, produced 377 g per plant in the poly house and 514 g per plant in the open field. In this case, both types of micropropagated plants are the same mericlone, from the same meristem. When daughter [runner] plants of the same mericlone were cold stored, the yield was 495 g per plant in poly house and 512 g per plant in the open field. All these plants originated from one apex so I cannot say the difference in production is due to a mutation or something like this but only to the physiological condition of the plant.

I have found several genetic troubles some of which I can recognize in vitro. With 'Aliso', I have found plants that have a half leaflet completely yellow and a half leaflet completely green. The same trouble may be expressed as a yellow streak in the leaflet. However, plants showing the yellow streak have produced from the base of the plant new shoots that look completely normal and green. In this case it is possible to separate this sort of chimera and to get a new, good proliferation of normal shoots. The most important chimera that is quite impossible to detect in vitro appears only in the adaptation stage. Generally it first appears with half leaflets yellow and half leaflets green. The second leaf appears with one leaflet yellowing and the third leaf appears almost yellowish or white. After that, there are two possibilities. The plant dies or it survives but survives with all the plant green.

This afternoon we spoke about the influence of NAA in the modification of the chromosomal plate. With 'Primella' regenerated from callus on a medium containing 2 mg/liter NAA, I never observed any difference in the number of chromosomes, unfortunately for me because I am looking for a mutation. With bright field, it is possible to observe how the typical chromatids are linked. But I asked my colleagues, particularly involved in cytological studies, and they said that these chromosomes are nucleoligenic chromosomes, that is the type of chromosomes that produce nucleoli.

G. W. Schaeffer

I'd like to open the floor to the panelists first. Do you have any questions for Dr. Damiano before we open it to the floor? Anybody on the panel here? Dr. Krul?

W. R. Krul

The fact that the chromosome numbers are the same is usually taken as a good indication that there has been no genetic change but you can get mitotic crossing over and inversions and still have the same number of chromosomes. There was a recent article [Evans, D. A., and E. F. Paddock. 1979. Mitotic crossing over in higher plants, pp. 315-351. In W. R. Sharp, P. O. Larsen, E. F. Paddock, and V. Raghavan (eds.), Plant cell and tissue culture - principles and applications. Ohio State University Press, Columbus.] which showed mitotic crossing over in tomato and the symptoms looked very much like the variegations in the leaves. So this might be symptomatic of this phenomenon.

G. W. Schaeffer

Anybody in the audience?

W. C. Anderson

The question I have is did the plants that originated from the aberrant plant produce normal fruit? In our lab, we have propagated quite a few clonal lines of strawberries without any difficulty until this last fall when we started in November to increase a lot of 'Hood' strawberries. We wanted to increase it up to about 5000. We obtained in that aberrant plants similar to what you had in your pictures at the rate of about 2%. And we were kind of disturbed about that.

S. Hvid

I'd just like to ask you if you could explain how the frigo plants are treated and why do you have a higher yield in the frigo plants than in the other ones?

C. Damiano

Dr. Anderson, you asked me if the plants produced normal fruits. If I consider the shape of the fruits, the habit of the plant, the time of blossoming and so on, I have to reply that certainly if I put 'Gorella' in vitro, I obtained the same cultivar without doubt. But for this behavior, this habit that the plant has because of the in vitro micropropagation, now I am not able to give you a correct answer. I am not able to distinguish if the physiological behavior can hide a mutation or a

modification in the habit and behavior of the plant. Regarding the second question, how can I explain the heavier crop in the frigo plants? This is a very delicate question because I can give you only one idea, only one opinion I have. I think that it depends generally on the cultivar. You have seen with 'Aliso' that there is no difference between fresh plants from either micropropagation or runners. Regarding 'Gorella', I think that the chilling requirement is not satisfied because now with micro-propagated plants, we plant our nursery in April, so the mother plants do not spend a winter. Then the mother plants produce runners and these runners are cold stored. If you plant the nursery before the winter, the mother plants spend a winter and you collect your runner plants and cold store them. So probably they are in a different physiological condition. This is only my opinion. I'd like to ask Dr. Scott if it is possible that this happens.

D. H. Scott

I think that would be as good an explanation as I could give, so I would agree.

M. Faust

In the table you showed, there were obviously quite large differences in the numbers - 370 for the micropropagated plant versus 400 or 500 for the others. Yet the letters in the table, which I assume represented Duncan's test, were all "a" which means no significant difference if I interpret that table correctly. How do you account for that? [See Table 4 of C. Damiano, strawberry micropropagation, p. 20 of these Proceedings.]

C. Damiano

There are some significant differences in fruit production for open field cultivation, but not for plants under plastic. In the open field, production per plant of fresh micropropagated plants was greater than fresh runner propagated plants. Fresh micropropagated plants produced more fruits than runner propagated plants. The same thing happened with cold-stored plants in open field. Cold-stored micropropagated plants produced more fruit than the standard plant. The size of the fruit is another thing. While fruit of the fresh micropropagated plant is smaller, the fruit of the cold-stored plant is the same size as the standard material but the weight is larger.

Voice

What is the difference between that and fresh micropropagated plants?

C. Damiano

Fresh micropropagated plants are the first runners produced in the nursery from the micropropagated mother plant (coming from the culture jar) and the fresh standard plants are the fresh runners from standard mother plants. Now for plants covered with plastic in January when the weather is still cold, the micropropagated plants, I mean the plants directly from culture jars, produced 207 grams, fresh micropropagated plants produced 377 grams and cold-stored micropropagated plants produced 495 grams per plant. For open field cultivation, micropropagated plants

yield 338 grams, fresh micropropagated plants yield 514 grams and the fresh standard plants 479 grams. This last difference is significant at 95%. And if you compare cold-stored micropropagated plants with cold-stored standard plants, the same difference is 512 grams versus 435 grams which is significant at 95%.

Voice

Yes, but that means that the micropropagated plants produce better.

C. Damiano

Yes. The only difference is in the size of the fruit because fresh micropropagated plant seems to still have the consequence of a juvenile phase. You see in the open field, 8.8 grams per fruit, and in the poly house, 10 grams per fruit. But this behavior is completely inverted if you consider cold-stored micropropagated plants. Here the differences are not significant and the fruit size is normal. And 14 g per fruit is a very good size for 'Gorella'.

Voice

Now just one thing. Did you count the number of fruits?

C. Damiano

I didn't count all the fruits. For average weight, I counted only 50 fruits at random.

Voice

In the micropropagated plants, the bottom line, did they have more or less fruits?

C. Damiano

Certainly they have more fruits.

Voice

You have more fruits that weigh less but give a lower total yield?

C. Damiano

Probably yes.

G. W. Schaeffer

I think we should move on to our next panelist. Dr. Scott, what is your perspective on genetic stability and how to deal with it?

D. H. Scott

If you go back into the early literature, you will find about 50 or 60 years ago a discussion by Bunyard in which he pointed out that the strawberry plant is notable for having very stable genetic make-up and this, I think, seems to apply to some extent in this micropropagation as we see it in strawberries. But even so, when large scale rapid micropropagation of strawberry plants is done by repeated recycling, one of the first questions that arises is whether there could be changes in the characteristics of the cultivars. Since strawberries have already

been propagated by the millions now by micropropagation, this has already become a controversial topic in strawberry propagation. There is one company that grows strawberries in Spain that has stopped using the micropropagated strawberry plants because they think they are getting results that are not characteristic of the variety. Actually, as we have observed some of that work, or more in line of talking with people who have observed it, there is a question about the physiological condition of those plants and how they are being handled. But I am going to address myself now to just two chimeras that we have seen in the propagation work at the Zanzi Nursery at Ferrara, Italy. One of these is the green-yellow type variegation that Dr. Damiano has just mentioned and we didn't, in this case, get any counts. It was in 'Belrubi' and occurred only the first year. But in 'Aliso', we saw a little different type of variegation than what Dr. Damiano has shown. It is a white streak and I'll have slides of both of these in just a moment. There we were able to take counts and, in 1978, out of 3,550 plants that were propagated directly by micropropagation, 72 exhibited the white streak. Now that wasn't plainly visible until the plants had been put in the greenhouse. They were taken out when that was observed after they had been in the greenhouse about a month and we saw no more of that type variegation later in the season. Then in 1979, again in 'Aliso', the same thing appeared with 40 plants present out of about 3,900. However, those are the only two cultivars in the work at Zanzi's that have shown any variegation. We have propagated about 60,000 plants of 10 other cultivars, none of which have shown any variegation. Also, there's another point to be mentioned and that is that in strawberries, there is a genetically unstable gene called June yellows that we see from time to time in breeding material, in selections, and in cultivars. In fact, it was the disorder that stopped the use of 'Dixieland', of 'Climax' in Europe, and of a few others. So far, in all of the material that I've had to chance to observe from micropropagation, I haven't seen any of this in the micropropagated plants. That's as much as I have to add.

Oh, yes, there are two slides. This is 'Belrubi' with the type of variegation that Dr. Damiano had seen. As we grew these plants, the one on the right continued to turn more yellow and finally died. The other became green and survived. Then the next slide will be the white streak or white pattern that we saw in 'Aliso' and this we have not detected in the greenhouse in the very small plants. We see this after they go into the greenhouse.

G. W. Schaeffer

Panelists, comments? Audience, questions?

M. Faust

Don, are those plants in the second slide - there are a number of what appears to be daughter plants or runner plants - are those from the plant which had the white streak in it?

D. H. Scott

Yes, they are. Many of the runners will show none of the white streak.

M. Faust

How can you call it a mutation then?

D. H. Scott

Because I think it is a very small sectorial chimera that disappears. You just don't happen to have runners being produced from that portion of the crown.

M. Faust

Really then, it doesn't have any consequence.

D. H. Scott

To me, it is not anything to be concerned about particularly, except that you need to keep it rogued out of the stocks. If a grower saw some of those plants in his field, he'd be suspicious probably that it was virus.

G. W. Schaeffer

Any other comments or questions?

Voice

What is the possibility that it is a virus that causes that sort of a chimera?

D. H. Scott

This would be a little difficult to answer except that so many of the runner plants do not show the symptoms at all. If it were a virus, you would expect by the time you had as many runners as these plants have, that some of the runner plants would show it. In a few cases, they do but many of them do not.

J. Rowe

One of the problems we have with variegations in culture is the fact that when you are culturing the plants, you can't actually see the variegations. I was wondering if you think this variegation pattern is the result of one event or several.

D. H. Scott

I couldn't hear all of that question.

J. Rowe

Well, when we are culturing the plants, in vitro, it is quite likely that an event can occur while you're culturing it and you are just maintaining that particular event through your cultures and it can build up in sizeable numbers in your line. And I was wondering with these 72 or 40 plants that you saw - do you think these were independent events or were they just maintained in the culture line?

D. H. Scott

We have no way of knowing because, as you say, it is very difficult to see some of these in culture. So I don't know. There is a good possibility that that was one of the very few that had it from the beginning and it was just propagated during the recycling, although these were only recycled three times.

P. Fridlund

The same type of chimera occurs occasionally in fruit tree seedlings. We see this when we grow different types from seed. In my own experience, I have seen it in peach, Prunus tomentosa, apple, pear, P. mahaleb and P. avium. So this is not an unusual thing although it doesn't happen every day. The symptoms themselves do not resemble virus symptoms although in these cases in strawberries, these were not proven nontransmissible. I would really doubt that they are transmissible. Now when you get a fruit tree seedling that grows up, say maybe 1 to 2 feet high and has this sort of thing, typically it will be on one side of the seedling and the leaves in one plane straight up will perhaps be white. As you go away from this plane, you will have half white and half green with the white half of the leaf toward the plane where the pure white ones are. Now when you take the buds adjacent to these leaves and propagate them, you get almost the same thing back as what you had in the beginning. By that I mean, half and half or all whites or all greens or whatever.

G. W. Schaeffer

We will move on to the next topic then. We will go to John McGrew, continuing in the strawberry vein.

J. McGrew

The third edition on strawberry comes up. As a plant pathologist, I figured that perhaps the thing to do would be to back up and look at the background level of variation, genetic or otherwise, in strawberry. The clonal selection studies in England in the 1940's demonstrated the major role of viruses and, to a lesser degree, the role of other diseases and nematodes in variation within a cultivar. The physiological status of the planting stock can have a marked effect on the first year's growth of a planting and, by affecting the plant density, can have considerable effect on the second year. Hondelmann's work on trying to detect genetic variation found that almost as important as the genetic were the environmental factors, location of the planting, climatic variation between years, which produced differences as marked as genetics [Hondelmann, W. 1965. Investigations on breeding for yield in the garden strawberry, Fragaria ananassa Duch. (in German) Z. Pflanzenzuchtung 54:46-60]. Furthermore, the rapid and continuing changes in the catalog of strawberry cultivars may conceal the potential for natural mutations in strawberry. Now there are a few examples, very few, of variability in strawberries and these are the controversies between 'Howard 17' and 'Premier', between 'Midway' and 'Early Midway', and between the original variegated 'Blakemore' and the non-yellowing Arkansas strain of 'Blakemore'. These may be mutations. They may also be simply the substitution by seedlings of the original clone. Now my remarks on the

strawberry will be limited to strawberry explants taken for the production of clones free of known viruses. The medium that we used up until 1975 was supplemented with 15% coconut milk. Since then, no auxins or cytokinins have been used. We're working strictly with plain, uninfluenced meristems of strawberries. These explants do not form callus. There are no adventitious buds, nothing approaching an adventitious bud. On occasion, I will leave a leaf petiole and leaf blade that have been incorrectly dissected and put in the medium without any axillary bud at the base. It may root, it may grow for a while, it may enlarge but it does not produce an adventitious bud. Now we have had some major "mutations". 'Dabreak' became 'Headliner' and that was mislabeled source plants. Both 'Sparkle' and 'Fairfax' became 'Suwannee' in the screenhouse because the virus-free 'Suwannee' was so extremely vigorous that it not only grew into the plot next to where it was supposed to be but across the aisle. One 'Midway' became 'Midland' and I think this was simply the tedium of dissecting out tips. But between 1966 and 1973, 52 lots of about 20 tip culture clones were sent out from Beltsville. So far as I've heard, these were all acceptable as the variety. We've not noted any major differences among the tip culture clones within a variety. There have been some differences in vigor. Now we put out the clones in the fruiting field and we put out the clones in the screenhouse and sometimes they appear at other places in the field. These differences in vigor between clone 1 and clone 2 of a variety may be reversed in the other planting. They are as often reversed as similar. There doesn't seem to be any marked difference in vigor. We also are comparing plants of extremely different physiological state. Some of these are being put in the screenhouse and field just as soon as they are produced newly rooted from the explants. Others just missed it last year and they have been sitting around for 11 months in a small pot waiting for the next planting season. This can make a lot of difference in the way they perform. During the last 4 years we have produced several hundred tip culture clones of about 150 cultivars and selections. We set out two-plant plots of many of these; 230 of these fruited in 1979, 180 more will fruit this spring. Except for slight differences in vigor and an occasional 'Midway' - 'Midland' effect, they seem to be remarkably stable. I've been hoping to select a best clone of each cultivar for further propagation and so far I just don't know if a best clone exists.

G. W. Schaeffer

Panelists, comments or questions for Dr. McGrew.

R. H. Zimmerman

John, earlier Dr. Damiano was discussing this point of the best clone and that was about regional adaptation of some of the European cultivars. If the meristems were taken in Belgium, plants produced in Belgium from those meristems did not do well in Spain or Italy whereas other plants of the same cultivar produced from meristems taken in Spain or Italy seemed to do better. Perhaps he would care to comment on that.

C. Damiano

The only thing that I can say is that the meristem should be collected from the plants that grow in the region or the area for which you are

producing meristem plantlets. That's the point. Because probably with 'Gorella' in Italy, we have a Belgian clone. Probably this clone is adapted to Belgium. Certainly Boxus collected these meristems from the best plants that he had in Belgium. We observed that if we collect the meristems from the best plants of 'Gorella' we have in Italy, also, the results, the production is different. So I suspect that we should collect the material, the explants, directly in the region, or in the field, or in the area or department for which we are producing the material. This is only a suspicion that I have. This should be done in the same way that we do in the breeding program, where we do the screening in Italy. For example, in the south we select plants adapted for the south, and in the north for plants adapted to the north. That is all.

D. H. Scott

Now I'd like to ask Dr. Damiano, though, in that case, where there were apparently differences, could that have been differences in viruses that are not detectable by ordinary indexing.

C. Damiano

Well, if I've understood, you've asked me how can I detect the differences because of the viruses that I cannot detect. This is the question?

D. H. Scott

No. My thought is that these plants that have been micropropagated from two different sources came naturally from different meristems and that it is very possible that there was a virus present in one that was not present in the other that could not be detected by ordinary indexing.

J. R. McGrew

The second question would be - did the best Italian clone of 'Gorella' do better in Belgium than the best Belgian clone in Belgium?

C. Damiano

Well, I don't know. The question is more complicated really. We know the problem involves another aspect of micropropagation, which is how many subcultures can we get with our material? Because I observed these differences in two different mericlones but one was propagated through 17 subcultures and the other was only 3 subcultures. This is another point. Well, at this moment I cannot say anything because we are just starting to test, to carry out a severe trial only on this aspect of micropropagation. And I consider that we have to carry out this trial because it could be a difference.

G. W. Schaeffer

Any further questions or comments? Then Dr. Krul will you give us your perspective on genetic stability.

W. R. Krul

I would like to recommend for people who are interested in genetic stability to read an article by Meins and Binns [Meins, F., Jr., and

A. N. Binns. 1979. Cell determination in plant development. BioScience 29:221-225.] in which they discussed the stable determined state. Basically what they're dealing with are things that most of us have seen in nature but have not really thought much about or fully comprehended. Their basic premise is that a cell or plant perceives a stimulus that we can call an inducer and this inducer changes a phenotypic characteristic of this cell or of the plants. Now you can remove the inducer and the phenotypic expression is still stable, it manifests itself. It will only change when the cells or the plant perceive inducer number two and the process is reversible. But as long as there is no other inducer, the plant remains in a physiologically stable state that is different from another state. I'd like to illustrate this with some of the things we've done with tissue culture in our lab. As far as the grapes go, I mentioned earlier in my talk that we were doing chromosome counts. Barlass and Skene [Barlass, M., and K. G. M. Skene. 1978. In vitro propagation of grapevine (*Vitis vinifera* L.) from fragmented shoot apices. Vitis 17:335-340.] did chromosome counts and generally when shoots are derived from adventitious buds, you suspect you'll have a higher frequency of genetic abnormality. They saw none in the chromosome counts that they made. The grapes were all  $2n = 38$  chromosomes.

I have some slides. I just heard a few minutes ago that there was a rumor spread about the vineyard that we established from somatic embryos in Maryland. The rumor was something to the effect that there was a tremendous heterogeneity, poor vigor or poor quality or something to that effect. I wish to put that rumor to rest right here and now. Cuttings from the parent clone of 'Seyval' show quite a bit of variation in the breaking of buds and the growth of buds. Cuttings taken from vines that were propagated from somatic embryoids are more vigorous and are more uniform in bud break and growth. If you doubt my word, you can take a 60 mile trip up to Montbray Vineyards in Silver Run, Maryland, and you can observe them for yourself.

Now, I might mention that the parent and the cloned vines are not similar. From what I've heard from Dr. Mowbray, who is growing the vines, the fruit cluster shape is slightly different, the stems on the cloned vines have red coloration whereas the parent vines do not, and the cloned vines are quite a bit more vigorous than the parent vines. Moreover, when Dr. Mowbray checked the description of the hybrid, he found that the cloned vines fit the description of the original hybrid better than the parent vines from which they came. So apparently the parent vines have been modified over time through asexual propagation. Someone, I think Dr. Janick, mentioned to me that possibly a sport of 'Seyval' was propagated and multiplied in America and, perhaps, that regeneration of some cells underneath the sport led us back to what we might call the original hybrid. John McGrew might have some comments on this. The other possibility is that when we went through callus culture, we've lost viruses through the cloning process. There are several reports in the literature that indicate this is possible.

Let's change the subject from grapes and continue on to the next. Judy Myerson, who did the grape work, took on a special project under my direction. She regenerated the velvet plant, *Gynura aurantiaca*, from callus. One of the first things we noticed was that the regenerated plants were much more vigorous and the leaf shape was substantially different. The parent plant had roundish leaves whereas the regenerated plants had leaves that are pointed and highly serrated. In addition, the tissue cultured plants were more vigorous as measured by plant height. Both types of plants were propagated by 3 cm cuttings. The cloned plants maintained their vigor through this first phase of asexual propagation, and were a little bit more uniform. Flowering was also more uniform in the cell cultured plants. Eighty percent of them had flowered at the time data were collected whereas only 25% of the parent plants had flowered. Further, one parent plant had gone all the way to seed while all the rest hadn't even formed flower buds yet. So there is quite a bit of variation in the parent plants but the cloned plants are remarkably uniform. Thus I think again we may have lost a virus or we may have captured some stable epigenetic state. I might also mention that axillary bud breaks in the parent plants are more frequent than in the clones. The clones do not sprout outside shoots as readily as do the parent plants. So we've changed the physiology of these particular plants, both the phenotypes, as seen by leaf shape, and by growth habit.

Something I'd like to impress on people who are studying embryogenesis is illustrated with some work I did at Beltsville using carrots. This has to do with the parent plant. Many of us were concerned how the parent plant was cultured and grown and the effects of these factors on subsequent regeneration and behavior. Carrot is a vernalizable plant. Theoretically when you cold treat the mature roots, you induce a flowering stimulus which promotes subsequent growth when the plants are brought out into normal ambient conditions. What we did here was to culture tissue from fresh mature roots, induce callus formation, proliferate the callus and test it for capacity to form embryoids. We did the same with roots that were stored at 4°C for 30, 60, and 90 days. Roots stored at 4°C for 30 and 60 days were a little bit more competent with respect to embryogenesis than were those not stored but after 90 days of vernalization, the tissue had completely lost embryogenic competence. The morphology of the callus was quite a bit different, it grew a more rapidly, and the effect was stable for a period of more than 2 years. Nothing we did caused these cells to differentiate. So again, I think we induced some sort of stable epigenetic change that was maintained as long as another stimulus did not come along to revoke it. That's about all I have to say.

G. W. Schaeffer

Comments by the panelists?

M. Faust

BTTT, I didn't get it quite straight. With those two different leaf types of the velvet plant - is it mutation or just a different physiological stage?

W. R. Krul

We don't know about the mutation because we haven't done chromosome counts on that one.

M. Faust

If they just grow a little differently, there are many, many examples that plants grow differently in different environments. If you compare the Washington state 'Delicious' apple versus the East Coast apple, the former is elongated while the other is round and short. Yet this is not a mutation. You can pinpoint the location and the environment under which they grew by the appearance of the end product. So I really cannot accept that as a mutation.

W. R. Krul

I didn't say it was a mutation. I say it's a stable, differentiated state which means that another genetic system in that plant which was formerly repressed is now active and it's active in a stable manner for a long period of time. This is different from growing plants in Washington or here because you can bring plants from Washington and they'll become like plants grown here but all these plants were grown under similar conditions and you still have these physiological differences.

M. Faust

I can see that but they are only physiological differences.

W. R. Krul

I'm not ruling out genetic differences. We haven't done that yet.

R. H. Zimmerman

Would you think this is comparable to the difference between a juvenile and mature state in some of the woody plants?

W. R. Krul

The article by Meins and Binns goes into the transition from juvenile ivy to the mature form. That's one of the examples of a stable differentiated state. And it can be reversed if you apply gibberellins. So that's the other inducer that brings back the original state.

W. C. Anderson

Have you ever tried propagating those plants from cuttings and seeing whether they still maintain that leaf shape?

W. R. Krul

Yes. That was the graph that we showed. Those were cuttings from cloned plants and cuttings from the regular parent. We've also taken seeds but they haven't germinated yet. Normally if you go through a sexual cycle, you lose an epigenetic or induced state. So if this is an induced state, in the *Gynura* at least, the seeds that we grow should come back as the parent. That's one way we could test that idea.

W. C. Anderson

I have one comment. On cabbage, we started from flower buds. A crop was put out and it bolted in the fall while normally it requires a vernalization period. That plant has been recultured and sure enough the plants from these recultures don't have a vernalization requirement. We've got to go through seed and find out whether or not those plants will act truly as annuals or whether the seedlings will be biennial. But the statement that was made this morning about plants started in culture from adventitious propagation are juvenile may not be completely true.

W. R. Krul

No, that's possible. With grape, we have a definite juvenile state. The leaf morphology is different, and the leaf morphology changes as the plant grows. Also the tendril formation doesn't occur until the 13th node on regenerated grapes which is sort of a juvenile characteristic of seedlings also.

R. N. Lawrence

Two years ago we were developing a system for high frequency propagation of celery using an embryogenic system we had developed. The question we were interested in answering was whether or not somatically formed embryos were similar to zygotically formed embryos, especially in terms of growth requirements. The objective was, of course, to produce plants with certain characteristics which you would then sell. When we used seed derived plants as a control and grew them in a Speedling-type of system, we found there was a distinct difference. It wasn't until we checked in our last step, where we took somatic embryos and put them in a minimal medium and surface sterilized seeds and put them in a minimal medium, that we found a comparable effect. The interesting thing is that the last stage of culture had a lingering effect on the growth and development of the plants as seedlings. And this was a minimal medium - this was where we came off the hormone medium with the embryos into a minimal salts medium. It is important I think to do the correct control. Of course, with your situation it is difficult because you don't have seeds to work with yet.

G. W. Schaeffer

Other questions? I might add in connection with this some work we've done with cereals, Bill, that plants regenerated from tissue-cultured rice were much more temperature sensitive than the original parent type. One wonders whether you might not be selecting for changes in membranes that perhaps don't show up so easily but which show up under temperature stress. Moving on down the list, Dr. Zimmerman is the last one on the discussion panel this afternoon. Dick, how does genetic stability look to you?

R. H. Zimmerman

I think we've covered most of the topics pretty thoroughly so far this afternoon. I will say that our results in strawberries have been similar to those of Dr. Damiano's and Dr. Scott's with regard to the variegation appearing. I heard it mentioned this morning that strawberries are

produced from axillary shoots. This is true, but they're also produced from adventitious buds as we found this year. Now, I don't know if this is just because we looked harder, but we didn't change our technique at all this year. Looking back at some photographs we had taken in previous years, on some of the clumps there is tissue that looks very suspiciously like adventitious buds. This year we found buds formed on the tip of the leaf so there is no question that they are adventitious. I don't know if this is a problem or not but I think it's something to keep in mind because this is the technique that is being widely used - the Boxus method. We followed it pretty faithfully.

As for the other plants that we've been working with, I showed you the one plant of thornless blackberry in which we had variegation. We've seen nothing in apple or blueberry to this point. However, you should keep in mind that we get most of our new apple cultivars from bud sports. There are very few apples introduced into commercial production that are derived from breeding programs. Most of them are bud sports found in the orchard by growers. Many of these are bud sports of 'Delicious'. So we're working with a system that may not be too stable to start with. We don't know. And this brings me to the point that I would most like to make. That is, every time we see a slight change, an aberration, or something we don't understand, in plants derived from tissue culture, we should not automatically suspect that it is the tissue culture that did it. There are many reports circulating but it is all secondhand. When you try to pin it down, well they have the data, its very good data but they don't want to publish it. This, I think, requires us to be very careful in what we say. Maybe these reports are correct but I think we need to evaluate very carefully the results of some of the studies where someone says we grew 500,000 micropropagated plants and they turned out to be no good at all and so the whole technique is no good. And then have that carried on, spread around the world, in effect, and then use this as a means for beating down the technique. I think we have to be very careful of this.

G. W. Schaeffer

Any further comments for the panelists?

D. H. Scott

There is just one more comment I'd like to make in connection with fruit plants, and that is that in some of the Rubus material, particularly the trailing type of cultivars that are grown on the West Coast, but also a few that are grown in the southern part of the United States, there are good data showing that there is genetic instability in the material. And certainly if that material is micropropagated, you can expect variations, differences.

G. W. Schaeffer

Are there any further comments or questions from the audience?

B. Briggs

Dick, on your blueberries, I was quite concerned. We noticed two years ago on 'Brittania' rhododendron, that small plants developed on the

leaves of that particular plant but since then we really haven't noticed it. We really didn't know where it came from but it was phenomenal to see it. My question would be this on blueberries. We have been very concerned on all the production of all plants that we try to eliminate anything that resembled callus. Now if you're working with a blueberry and you are creating some callus formation from that leaf and then you're getting plantlets from that, do you have any research to indicate that those plants coming from callus are not as stable as those coming from the normal shoot tip development?

R. H. Zimmerman

We can't answer that yet, Bruce. One thing that we want to do is to isolate the adventitious buds and grow plants from adventitious buds, plants where the adventitious buds form from the callus, and plants that grow from axillary shoots and compare them. But the shoots have to be isolated at a very early stage and then cultured and we'll probably have to culture them on a low or no hormone medium to make certain that there is no further proliferation. We just haven't been able to do this study yet.

## Meristem Culture for Production of Virus-Free Strawberries

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The role of viruses in contributing to the running-out of strawberry cultivars has been recognized for over 50 years. Some 25 viruses or virus-like diseases (3) have been described which range in their effect on cultivated strawberries from negligible to lethal. The most insidious effect is a decrease in vigor without any distinctive or obvious symptoms, resulting in lower plant production in the nursery and reduced yields in the growers field.

The discovery at East Malling in 1942 (5) that Fragaria vesca L. was a sensitive indicator of several viruses which are latent in most cultivars provided the means to select more vigorous and productive clones of many cultivars.

Heat treatment of entire plants for one or more weeks at 35° to 40°C proved effective in eradicating certain viruses, e.g. strawberry mottle. The technique of excising small axillary buds that formed during or shortly after heat treatment provided a method for producing stocks free of some additional viruses.

The use of tissue culture in combination with heat treatment was pioneered in 1962 (1) and has proven effective, in combination with improved indicator clones of F. vesca and F. virginiana, in producing clones of most cultivars that are free of all detectable viruses.

Since 1945, our laboratory has been providing the strawberry industry with strawberry stocks free of known viruses. As techniques to identify additional viruses have been discovered, a reworking of these stocks has been necessary.

At present, all known viruses, except one, can usually be eliminated from strawberry cultivars by use of tissue culture without heat treatment, provided the excised tip is less than 1 mm long. The technique is not infallible, so regardless of the size of the explant, the clone must be reindexed to demonstrate absence of viruses.

Procedures used at Beltsville. A candidate plant is potted in the greenhouse and indexed by excised leaf grafts (2) to the East Malling clone of F. vesca (EMC) and to F. virginiana clone UC-10. If either indicator shows any symptoms, the meristems of runner tips from the candidate plant are excised to tissue culture.

The soft agar medium contains half strength Knop's salts plus vitamins and minor elements (Table 1), but no growth regulators. The length of each explant is recorded, and those that grow into plantlets are transplanted into sand under mist and given a clone designation. This phase requires 2 to 6 months.

Once established, each clone is transplanted into soil on the greenhouse bench and reindexed. This phase requires at least 4 more months. Those clones that prove to be free of viruses are propagated by runners for field-fruited trials and possible screenhouse propagation and additional runner tips are excised for storage of virus-negative clones in tissue culture.

When these explants have rooted, they are allowed to grow until several leaves have developed and then are held in an illuminated incubator at 40°C (7). Plantlets generally remain in good condition for a year and are readily transferred and reestablished for further storage. Clones held in vitro are isolated from insect virus-vectors, soilborne pests, and bacterial and fungal pathogens.

Some general information, based on several years' use of the tissue culture technique and over 7,000 excised tips made at Beltsville, should be of interest to others who may be considering this technique.

Method of dissection. Both intercalary and terminal runner plantlets are collected in the greenhouse, dipped briefly in about 0.1 percent sodium hypochlorite and placed directly on a piece of absorbent paper to remove surface moisture. The replaceable blade scalpels and fine watchmaker's tweezers used are flamed in alcohol. Dissection is done in a sterile room under a low power (15 X) microscope and explants are measured with an ocular micrometer as they are transferred to the culture bottle.

Early attempts to dissect out strawberry meristems by peeling back each layer of clasping stipules made it obvious that there had to be an easier, less hairy method. I now approach the meristematic area from below, by holding the distal end of the runner plantlet and making thin, transverse slices until a cone including the apical dome and one or two embryonic leaves can be teased out. This permits some control of the size of the explant that is removed. A comfortable rate of about 20 explants per hour can be achieved.

Contamination. About 20% of excised tips carry microorganisms, mainly bacteria. This rate varies somewhat by cultivar, but mainly by season. It is lowest in the spring, increases through the summer and is variable by late fall and winter. Size of the explant seems to have no relationship to the contamination.

Relationship of explant size to rooting. There is a consistent relationship between explant size and successful rooting shown in Fig 1. These percentages are for explants taken throughout the year and exclude

explants that were contaminated or improperly dissected. Included are explants from both heat-treated and non-heat-treated sources.

Relationship of explant size to freedom from pallidosis. In the early attempts to eliminate pallidosis (4) from strawberry stocks, a combination of heat treatment, up to 50 days at 35°C, followed by tip culture was used. It soon became obvious that the only factor determining success was the size of the explant. For the last 2 years, prior heat treatment has not been used. Fig. 1 shows the effect of size on the percentage of explants from pallidosis-infected sources that indexed free of this disease and points out the necessity for reindexing all such tip-clones.

Relationship of explant size to efficiency. If we combine the two previous figures, the advantages of taking explants in the size range between 0.5 and 0.9 mm becomes clear (Fig. 2).

Other viruses. There have been viruses other than pallidosis in several of the candidate plants in this program. The number of explants of any one has not been high enough to determine the relationship between explant size and successful virus elimination. Mottle, crinkle, and vein-banding have been eliminated from some explant cultures. One exception has been chlorotic fleck (6) reported from Louisiana and which was present in some plants of 'Tangi' and in all plants of one selection received from there. All explants taken down to 0.3 mm, continued to index positive for this virus. Studies combining heat treatment with small tips are under way and results look promising.

Indexing. The procedures are relatively simple. Establishing indicator clones and learning how to grow them takes some experience. Symptoms of some viruses are masked during hot weather. Under the best of conditions, symptoms may be mild or transitory.

#### Summary:

The origination of explant clones free of virus from field-grown stock requires a 1- to 2-year lead time before rapid propagation can be justified. Indexing of clones after the first tip-culture stage and fruiting of sister-plants are prerequisites.

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Table 1. Culture medium for strawberry tip culture

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|   |
|---|
| Macronutrients, modified Knops x0.5 (mg/liter)  |
| Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O (500), MgSO <sub>4</sub> ·7H <sub>2</sub> O (125), KH <sub>2</sub> PO <sub>4</sub> (125)         |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (125), KCl (125)  |
| Micronutrients (ug/liter)   |
| FeSO <sub>4</sub> ·7H <sub>2</sub> O (375), Na <sub>2</sub> EDTA·2H <sub>2</sub> O (375), H <sub>3</sub> BO <sub>3</sub> (30),                        |
| MnSO <sub>4</sub> ·H <sub>2</sub> O (80), KI (60), CoCl <sub>2</sub> ·6H <sub>2</sub> O (5), Na <sub>2</sub> MoO <sub>4</sub> ·H <sub>2</sub> O (2.5) |
| Growth factors (ug/liter)   |
| Thiamin (200), Ca pantothenate (20), biotin (20), niacin (1000)   |
| pyridoxine (200)  |
| Carbon Source   |
| Glucose (30 gm/liter)   |
| Agar (8 gm/liter)   |

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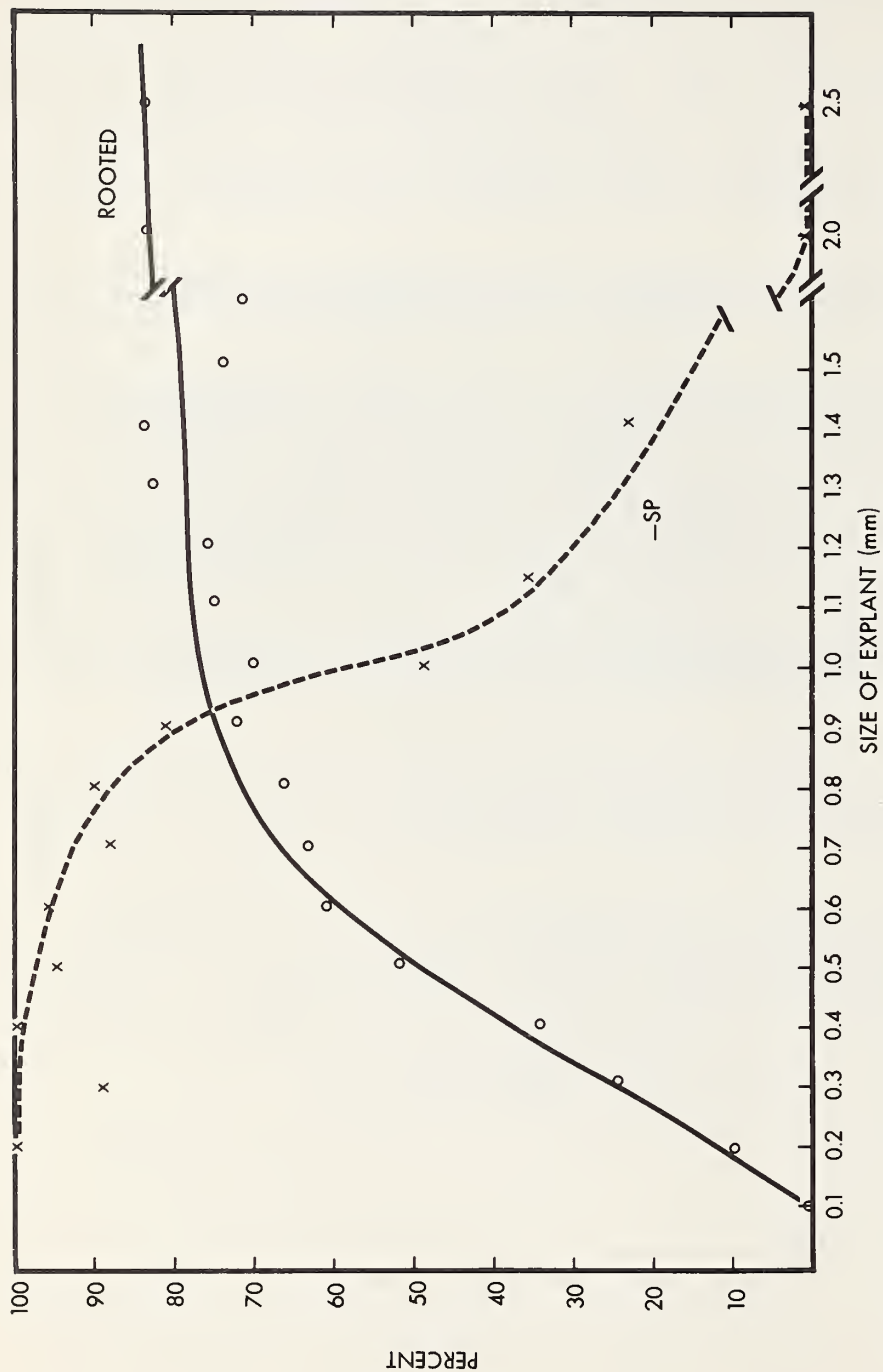


Figure 1. Relationship of explant size to successful rooting (N=1673/2954) and to freedom from strawberry pallidosis disease (-SP) (N=250/353):

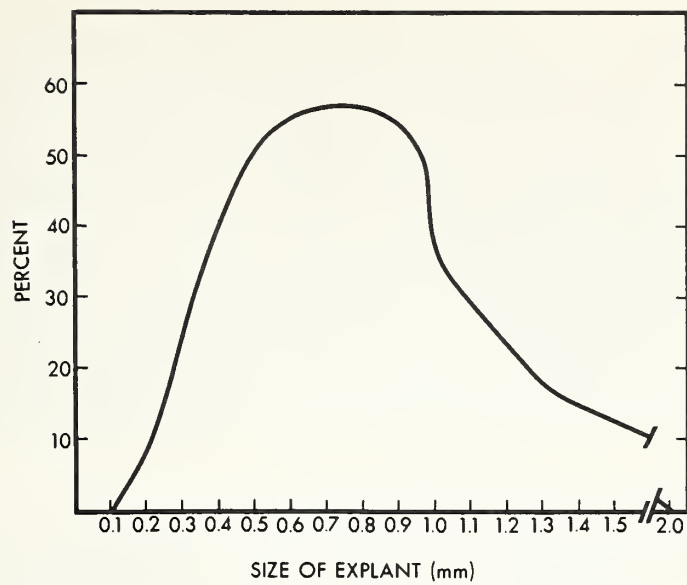


Figure 2. Efficiency (percent rooting x percent pallidosis-free) of production of strawberry tip-culture clones by size of explant.

# Maintenance and Distribution of Virus-Free Fruit Trees

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Abstract. The general objectives of virus-free fruit tree repositories are discussed and the extremely practical repository, IR-2, is discussed in detail. Although IR-2 may have limited use for aseptic tissue culture, and that use primarily as a substitute for thermotherapy in eliminating virus infections, other repositories that preserve germplasm as genetic resources would have greater use for the technique.

## An overview of virus-free fruit tree repositories

Most established and emerging nations have developed or are developing nursery improvement programs for producing virus-free citrus and deciduous fruit trees. The term virus as used herein functionally includes viruses, viroids, and those organisms that cause virus-like diseases. These pathogens are nearly always transmitted by grafting, and the greatest increase in disease incidence occurs from propagation and topworking with infected scions or rootstocks. Each nursery improvement program appears procedurally different because of the diversity of methods used, specific crops included, environment and personnel qualifications. Naturally with this diversity, program excellence varies from superior to deficient. However, the goal of producing healthy nursery trees is the same for all programs.

In the eastern European countries and some others, the programs tend to service the particular organization to which they belong such as state farms, cooperatives or similar producing units. Most programs in the western nations service the industry nationally. Among the larger programs are those of the United States (IR-2), Great Britain (EMLA), France (CTIFL), Canada (CDA) and Australia (FVF). Other fine smaller programs operate in Belgium, Germany, Netherlands and Switzerland. Most programs have a vertical structure in which the operations extend from initial accumulation of cultivars through various testing and repository functions to supplying propagation material directly to nurserymen.

Normally, superior clones of visually virus-free fruit trees are selected for inclusion by researchers, nurserymen, or other competent persons. Clonal superiority is determined by visual observations of past performances or by other measurements. Once the clone is obtained, it is propagated for the repository and simultaneously tested to detect possible virus infections.

The most commonly used testing procedure is to graft parts of the tree being tested to other woody plant cultivars that are sensitive indicators for known viruses. Such grafting transmits any virus that is present in the infected tree to the indicator in which characteristic symptoms occur.

A complete range of indicators can contain numerous cultivars. In a few cases it is possible to test for viruses by sap transmission to select herbaceous indicators. Other methods of virus detection in fruit trees are not developed sufficiently so that they can be used exclusively. For example, there are a few serological techniques that are excellent for detecting specific viruses, but most of these techniques have inherent problems preventing their overall use. Additionally, most fruit tree viruses have not been isolated which prevents the production of antisera. Except in testing for phony peach, a rickettsial disease, chemical tests are not very good. Electron microscopy alone confirms only the presence of characteristically shaped and sized particles and is not diagnostic. However, when it is combined with certain serological techniques, it has diagnostic applications.

Because viruses are nearly always transmitted to daughter trees during propagation, it is necessary to propagate with materials from virus-free sources. Occasionally this is not possible because there is no known healthy individual of the cultivar, the desirable mutant, or other valuable selection. Thus it is necessary to develop healthy scion and rootstock sources.

There are several techniques for developing healthy trees. The particular method used is not important if success is obtained. 1) The simplest method is to isolate virus-escaped tissues if such escapes occur in the particular host-virus combination of interest. Thus buds of apple trees without chlorotic leaf spot and sour cherry trees without green ring mottle viruses occur frequently on infected trees. It is easy to propagate a virus-free daughter tree with these buds. 2) Apomictic seedlings of citrus are usually free from infecting viruses, but these seedlings retain undesirable juvenile characteristics for long periods making this kind of virus-free development inconvenient. 3) Meristem tips (0.15 mm) are usually virus-free when isolated and grown aseptically, but this is a very difficult technique not adaptable commercially, and sometimes difficult to master even by scientists. 4) Chemotherapy has little or no effect on true viruses and virus diseases although it has been used to mitigate symptoms in, but not to cure, orchard trees infected with organisms causing virus-like diseases. 5) Thermotherapy is the only consistently successful, simple method that can be used to free fruit tree parts from viruses, but thermotherapy is not applicable for curing infected orchard trees.

Healthy plant material appears to be produced in two general ways by thermotherapy; apparent inactivation of the virus and the production of virus-escaped tissue. To inactivate the virus a diseased tree or part of a tree is placed in constant elevated temperatures, usually 37-38°C, for a period of 21 or more days. Buds from the treated trees are then removed and propagated. A percentage of healthy trees results. This technique is particularly effective for developing trees free from most isometric viruses and organisms. With the second method, new shoots are forced in the heat, and after 21 or more days, small tips (about 5 mm  $\pm$ ) are excised

and grafted to virus-free seedlings. Most of these small grafted trees are virus-free emphasizing that this technique is more effective than the previous one. Although most viruses can be eliminated with this technique, a few exceptions are not. Previously, a hot water dip for 5-20 minutes at 45-50°C was assumed to rid propagation materials of some viruses. However, this treatment is now generally believed to rid infected materials only of organisms that cause virus-like diseases.

Very few vectors of deciduous fruit tree viruses are recognized in North America which suggests that some of these viruses may not spread naturally. Apparently this is true. When natural spread of viruses occurs, the known vectors are usually pollen, eriophyid mites or nematodes. Pollen-transmitted viruses and some transmitted by eriophyid mites are difficult to control, and thus all trees in a defined area can become infected. Homopteran insects primarily transmit the organisms which cause virus-like diseases. These organisms frequently are controlled effectively by eradicating nearby wild hosts and roguing infected orchard trees. Nematode-vectored viruses sometimes can be avoided because this kind of vector is not very mobile although it may be omnipresent in some areas.

After virus-free trees have been produced under a supervised program and planted in an orchard, how long can these trees be expected to remain free from infection? Because increase of most viruses occurs during propagation, this source of infection has automatically been eliminated. Additionally, if adequate isolation from external disease sources is established through wild host plant extermination, then the orchard trees theoretically should remain healthy. Therefore, the internal exclusion of infected trees by planting trees produced under a supervised program, avoiding topworking, and providing isolation from external virus sources assures that many specific viruses will not spread into an orchard. However, some fortuitous exceptions may occur.

#### IR-2, a practical, virus-free, deciduous fruit tree repository

Although the overview of virus-free plant production as described is generally understood, the procedures and operational details usually are not. Therefore, an insight into the practical procedures used is appropriate here even though specific details may change frequently within a dynamic project.

Perhaps the best example to review during this symposium is IR-2 (Interregional Research Project #2) of the United States which is concerned only with deciduous tree fruits. IR-2 is one of the first and largest of the national virus-free programs. This project differs from other national programs because it functions only as a repository source for small amounts of propagation materials that are used for establishing virus-free foundation blocks. IR-2 does not supply materials in usable quantities for commercial propagation. The project also provides active support and training for State Department of Agriculture personnel who

supervise nursery improvement programs that produce virus-free trees and for interested commercial nurserymen and other persons. Obviously IR-2 has a strong plant pathological orientation.

The plant inventory of IR-2 contrasts sharply with those of pure repositories that maintain clones solely for their genetic characteristics. Normally IR-2 maintains clones that have immediate commercial and/or practical interest while avoiding clones that are of value only for their potential genetic resources.

The objectives of IR-2 can be categorized into three general topics: 1) To obtain apparently virus-free valuable cultivars and clones of deciduous fruit trees, verify their apparent virus freedom by extensive testing, maintain them in isolated repositories, and distribute small amounts of propagating materials from them for research or for release to industry; 2) To develop virus-free individuals by any method from cultivars and clones that have no known virus-free individuals; and 3) To do research on methods, techniques, viruses, and host plants in relation to improving the performance of the repository.

Suggestions of specific candidate clones for the IR-2 repository and their sources are normally received from interested scientists, state regulatory personnel and leading members of industry. Also specific suggestions sometimes originate from IR-2 personnel in response to anticipated needs for certain desirable cultivars or clones. After establishing the need for each clone, propagation material is obtained. To simplify and expedite processing, these materials are received only during the winter greenhouse season.

Every candidate clone of each genus is tested preliminarily immediately upon receipt to establish the presence or absence of certain viruses that experience frequently has shown to be present. Indexing is the term used for this testing.

All preliminary and most routine virus indexing is now done with woody plant indicators in a greenhouse. Formerly indexing was done exclusively in the field, but recently developed techniques have permitted increased accuracy, efficiency and economy by using greenhouse facilities. Additionally, the lag times for results have been reduced from one to five years in the field to three to ten weeks in a greenhouse. The principal method of indexing is a grafting technique called double-budding. With this method two pieces of the candidate tree are budded low on a healthy potted seedling, and a selected indicator cultivar is budded immediately above this inoculum. If a virus is present it is graft transmitted from the inoculum buds to the seedling rootstock and in turn from the seedling to the indicator bud. When the sensitive indicator bud grows, characteristic symptoms of a particular virus infection will be exhibited in the new growth. All indicator cultivars are selected because they produce strong symptoms in response to infection with a specific virus even though that virus may be latent or symptomless in most other cultivars.

Except for preliminary indexing, the various candidate clones are processed differently because of their anticipated virus content. The genus of the candidate clone determines the methods used.

Thus three trees of each stone fruit candidate clone are propagated on appropriate virus-free rootstocks in containers. They are grown under screen until they attain sufficient size to provide enough budwood for complete indexing. Meanwhile, if the preliminary indexing detects an infecting virus, the candidate clone is discarded and attempts are made to find a virus-free source of the same clone.

After attaining sufficient size for indexing (usually one growing season) one of the three propagated trees is selected for further intensive indexing. In *Prunus* this is done by double budding to three replicates of each of eight indicators in the greenhouse. If virus is detected the tree is rejected and the second and third tree may be intensively indexed. However, the second and third trees are indexed only when the viruses are known not to infect the source cultivar systemically. Otherwise the candidate clone is rejected and the search continues for a healthy source. However, if it becomes evident that healthy source is not obtainable, the infected candidate clone is made virus-free with thermotherapy.

When a tree of each candidate clone is determined to be apparently virus-free, it is designated the nucleus mother tree of that clone, and it is maintained permanently in a container in a screenhouse. Two daughter trees of each nucleus mother tree are propagated and planted in isolated repositories. It is from these daughter trees that most budwood is distributed. Meanwhile subpropagants of the nucleus mother trees are made in an experimental field to verify gross identities. Horticultural evaluations are not made because such evaluations may be valid only for the immediate area in which they are made.

Soon after beginning to accumulate candidate clones of apple and pear, it became evident that different routine procedures would be needed for these species. The high incidence of virus infection encountered made the procedures used for stone fruits repetitious and worthless. Thus, propagating materials of all pome fruits are now treated with thermotherapy immediately upon receipt as well as being given the preliminary advisory indexing. The trees produced by this thermotherapy are subsequently indexed annually for about three years. This indexing ensures successful elimination of the originally detected viruses before the trees are accepted as individuals of a candidate clone.

A single treated individual of each apple candidate clone is then selected for intensive indexing on four different indicators in the greenhouse and three in the field. Fruiting of field-grown indicators is required for detecting fruit-deforming viruses as adequate foliage indicators have not yet been developed for them. Similarly, thermotherapy-treated pear trees are selected and indexed on three indicators in the greenhouse and two in the field. If any of these indicators detect a

virus infection, that candidate is rejected and a different tree of the same clone is indexed thoroughly. However, if no virus is detected in the original candidate, that tree becomes the nucleus mother tree for that clone, and it is maintained permanently under screen as a containerized tree.

Two daughter trees of each nucleus mother tree are also propagated and planted in isolated repositories as budwood sources.

The demand for virus-free propagating materials has continually intensified during the succeeding years. Thus, budwood from IR-2 repositories has been sent to 40 states and five Canadian provinces. Canada is an active cooperator with IR-2 and also maintains a close administrative tie through membership on the IR-2 Technical Advisory Committee. Although some IR-2 clones are used for research, most have been used to supplement or initiate state-administered nursery improvement schemes. Considerable foreign interest in these clones also has developed through the United Nations, USDA AID and other programs. All of the clones sent to foreign destinations are done so via the USDA Plant Germplasm Quarantine Center so that they may be used as exchange material for valuable foreign germplasm.

#### Potential uses for tissue culture in fruit tree repository programs

The foregoing describes an extremely practical repository whose main objectives are not to preserve genetically valuable germplasm per se. Therefore, of what use is aseptic tissue culture to a project such as IR-2?

Superficially its application appears limited because the needs for IR-2 propagation materials require that they be immediately usable. However, several important uses for aseptic tissue culture appear to warrant its use in a project of this type. Consequently IR-2 has developed a small laboratory for adapting aseptic tissue culture techniques to its needs.

The most important need is for virus elimination from infected clones. Although thermotherapy is easier and more rapid, a few viruses, for example apple stem grooving, are refractory to heat and are rarely eliminated in this way. Additionally, some virus-infected peach and cherry cultivars are too heat sensitive to survive a normal thermotherapy treatment. However, viruses can be eliminated from these cultivars with meristem dome isolation. In certain instances virus-free, aseptically-cultured fruit tree germplasm could be used for crossing quarantine barriers more rapidly. Thus by definition and fact the viruses and other plant and animal pests would be automatically excluded from such aseptically-cultured cultivars. This technique was originally suggested for international exchange of citrus. Finally, under certain circumstances, aseptic tissue culture could be used as a replacement for some nucleus mother trees, but never for repository trees. However, as propagation materials are frequently harvested from the nucleus mother

trees for distribution, and very little labor and expense are involved in their maintenance, only a reduced space requirement appears to be an advantage for maintenance by aseptic tissue culture.

Among all types of repositories, those that preserve germplasm only for the potentially valuable genetic characters it contains appear to have the most use for aseptic tissue culture techniques. Most of their outlets are to researchers, and therefore, much of the material is not in immediate demand and could be preserved conveniently in a cultured form. Additionally, the uses for these types of materials often can be anticipated by the researchers well in advance. Therefore, sufficient lead time occurs so that the cultured clone can be developed into a usable form for transmittal to the researchers.

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The setting up of a laboratory for the industrial production in vitro of plants needs to be carefully assessed. In fact the initial costs are so high that there must be a large and regular demand if the undertaking is to be profitable. What has happened in Italy in recent years provides an interesting example. One of the main reasons for the construction of two strawberry plant producing laboratories in Emilia-Romagna - a region with 4,685 hectares of strawberry cultivation out of a national total of 10,493 - was to reduce the number of diseased plants. In fact, for a number of years there had been worrisome signs of a decline in crop yields. This was attributed to the poor health qualities of the plants. The problem was all the more worrisome in view of the fact that the intensive nature of cultivation in the area virtually ruled out the possibility of planting on new land so that there was the risk that the situation would continue to get worse year by year.

The same reason that convinced the Centrale Ortofrutticola alla Produzione at Cesena and the Zanzi nurseries at Ferrara to build their laboratories, which presently produce around 3 million plants per year (strawberries, rootstocks for apples, peaches, plums, cherries, etc.), at the end of last year led an association of growers in the Campania, in southern Italy, to build a laboratory for the production of strawberry plants. The plan is to produce 200,000 strawberry plantlets per year with two technicians. This appears to be the minimum number required for laboratory production with today's prices for micropropagated strawberries in Italy (18 cents = 150 lira). Obviously, this minimum number will be different for other species.

In order to outline the general principles underlying the design of a laboratory, I have taken the case of a production of around one million strawberry mother plants per year and shall illustrate some of the practical solutions adopted. Such a laboratory is sufficient to provide for the economical renewal of the nursery mother plants of a region producing 60,000 tons of fruit per year.

The laboratory has been schematically divided into separate areas corresponding to the various functions that are performed. In practice, some of the functions can be performed in the same room but in order to simplify the description it is best to consider them apart (Fig. 1).

Zone A, Heat Treatment Chambers - Indexing Glasshouse. These elements are not a part of the laboratory proper, but are nonetheless essential if the material produced is to be certified. The design and construction of the heat treatment chambers and the indexing glasshouses depends on the material to be controlled.

Zone B, Collection of Meristem Tips. This room should be fairly small ( $10 \text{ m}^2 = 108 \text{ ft}^2$ ) in order to reduce air flow as much as possible. It is equipped with a table, a stereoscopic microscope, and several pairs of forceps and scalpels of different sizes. It is also useful to have UV lighting to be turned on 12 hours before starting work in order to maintain sterile conditions (Fig. 2).

Zone C, Transfer of Explants. This is the room equipped with the laminar flow cabinets that enable explants to be transferred from one culture jar to another under sterile conditions. It must be large enough to allow the installation of five cabinets with a working surface 1.5 m long. Cabinets of this size enable an operator to transfer the explants into 100 to 120 new jars per day, enough to produce around 1,000,000 plantlets per year divided into two seasons. Each cabinet is equipped with one interchangeable blade scalpel, one pair of long-handled forceps, one alcohol or gas burner, cotton wool and a small quantity of alcohol.

Zone D, Growth Room. Each 500 ml culture jar with a diameter of 10 cm can contain an average of 40 rooted plantlets. One season's production can therefore be achieved with about 15,000 jars, the preparation of which requires about one month estimating a daily production of 500 jars at the laminar flow cabinets. The lead times for rooting (about 15 days) and acclimatization (45 days) mean that it is necessary to complete the transfer of the plantlets to the rooting medium 60 days before the date planned for open field cultivation. The growth room must be designed to contain at least 7,500 jars at the same time. This requires 80 to 90  $\text{m}^2$  of shelving, for which a room of 60  $\text{m}^2$  is sufficient (Fig. 3).

Special attention needs to be paid to temperature control in the growth room since its size makes it difficult to maintain a constant temperature on all the shelves. The air conditioning system must not create turbulence that would be bound to favor the contamination of the cultures.

It is necessary to prevent the air heated by the lamps from remaining blocked between the shelves. One solution to this problem is to make the air from the conditioner pass between the shelves close to the lamps (Fig. 3). It is absolutely essential not to have sources of heat, such as the ballasts of the fluorescent lamps, in the growth room. They must be mounted outside. An automatic device for turning off the lamps when the temperature rises too high must also be fitted, since high temperatures cause the greatest damage to the plantlets. In order to obtain steady growth of the plantlets it is sufficient (at least for strawberries, plums and some other woody species) to have a light intensity of 1,800 to 2,300 lux, provided by industrial white light fluorescent lamps. The zones B, C, and D described above represent what can be called the heart of an industrial laboratory. They are supplemented by a series of ancillary services which help to increase the flexibility and productivity of the laboratory itself.

The fact that in vitro explants can be kept alive at low temperatures (20°C) for several months makes it possible to schedule production fairly accurately. A 12 m<sup>3</sup> (425 ft<sup>3</sup>) refrigerator provides sufficient space for the regulation of one season's production. The refrigerator is used to store the plantlets before rooting and those ready for the acclimatization phase. Another smaller refrigerator with a more accurate temperature control is necessary for the in vitro storage of germplasm.

The glassware in common use in this type of laboratory (cylinders, pipettes, erlenmeyer flasks, beakers, etc.) is no different from that employed in research laboratories, though there is a tendency to prefer plastic material in order to reduce breakage.

The container of the medium is worth describing briefly. It is a normal 500 ml glass jam jar with a diameter of 10 cm to hold around 150 ml of nutritive medium. There are various types available on the market but the type chosen should satisfy two conditions: first, that the walls should be as straight as possible (so as to facilitate transplanting) and second, though less easy to find, that the lid should rest on a flange outside the jar (so as to reduce the risk of contamination). For the culture of apices and for the conservation of germplasm, normal laboratory test tubes are used. As can be seen, the glassware used is somewhat less sophisticated than that of a research laboratory, but the results achieved are excellent, since small errors are offset by the very large quantity of material produced.

It might be thought that the very high cost per liter of the nutritive medium was due to the use of hormones and vitamins, but in reality, even though they are extremely expensive, they are used in such small quantities that their cost is virtually negligible (Table 1). The greater part of the cost is due to the sugar (glucose or sucrose) and especially the agar used. The cost of \$1.07 per liter can be reduced by using commercial grades of sugar and agar for human consumption.

The consumption of demineralized water in an in vitro culture laboratory is very large, both for the preparation of the nutritive solutions and for the cleaning of glassware. The quality of the water supplied by mixed bed demineralizers is usually excellent with a resistance of at least 0.2 k $\Omega$ . When choosing the type of equipment, which will have to produce 8,000 to 12,000 liters of demineralized water per month, account must be taken of the salinity of the water supply. The presence of chlorine, if the water comes from the public water supply, and that of nitrogenous compounds, which can prevent the regular growth of plants, must not be overlooked.

Glassware Cleaning Zone. This is a separate room where the culture jars are carefully washed and rinsed with distilled water. These operations can be performed either by machine or by hand.

Media Preparation Zone. In order to prepare media rapidly it is useful to have mother solutions with a concentration 200 times greater

than that of the final solution. With one liter of such a concentrate it is possible to prepare enough medium for 1,200 to 1,500 jars. The media preparation room must be equipped with: 1 technical balance, 1 pH meter with the electrode protected from shocks and with a lead wire at least 150 cm long, and 1 or 2 25-liter gel preparation units (Fig. 4). An analytical balance is also necessary but, in view of its delicacy, it would be better to keep it in a separate room.

Sterilization Zone. This is the room containing the autoclaves. The horizontal type is the fastest and most convenient to use, but in Italy it costs more than three vertical double-wall autoclaves, which are capable of handling the same production and, above all, avoid the risk of production being completely halted in the case of a breakdown.

The rest of the laboratory consists of a storeroom, offices, and toilets.

When the rooting phase has been completed, the plantlets are ready for acclimatization and subsequent transfer to open field cultivation. Acclimatization usually lasts 40 to 45 days. It is necessary to continue with the long photoperiod and 90% relative humidity during the first 15 days in order to obtain fast and steady growth (Figs. 5, 6, 7).

Since the demand for strawberry mother plants is concentrated in the fall and in the spring, when nurseries do their planting, it is possible to multiply other species in the off-peak periods in order to improve the utilization of the laboratory's capacity.

An extremely important aspect of the setting up of a laboratory is the choice and training of the staff. It goes without saying that production will be greater with more highly qualified and better trained personnel. For a production of 1,000,000 plants per year two qualified in vitro culture technicians are sufficient. The rest of the work can be carried out by workers who have only received on-the-job training. Another worker trained in glasshouse techniques is necessary for the acclimatization phase.

Although an industrial in vitro culture laboratory has the facilities needed for research, it almost certainly will not be able to afford the investment required to develop a new process to the point of commercial production. It is therefore necessary that there should be close collaboration between the industrial laboratory and research institutes, which should provide assistance and inform the staff of new developments. I am well aware that there are difficulties inherent in this relationship due to the different nature of the two functions, but every effort must be made to overcome them if the best results are to be achieved.

It is equally certain that a propagation laboratory cannot always reverse a decline in crop yields since this may be due to factors such as poor cultivation techniques, unsuitable varieties, etc., which are unaffected by the propagation technique employed. Nonetheless, the use of micropropagated material goes a long way towards solving the problem.

Table 1. Cost of materials in 1979 for preparation of 1000 liters of shoot proliferation medium<sup>z</sup>.

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| <u>Item</u>                    | <u>Cost</u> |
|--------------------------------|-------------|
| Macronutrients                 | \$ 17.53    |
| Micronutrients                 | 3.00        |
| Chelate (Na <sub>2</sub> EDTA) | 15.00       |
| Vitamins                       | 14.41       |
| Indolebutyric acid             | 2.40        |
| Benzyladenine                  | 9.60        |
| Sucrose                        | 232.89      |
| Agar                           | 768.31      |
| Others                         | 6.00        |
| <hr/>                          |             |
| Total                          | \$1069.14   |

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Cost per liter = \$1.07

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<sup>z</sup> Converted to dollars at estimated exchange rate of \$1.00 = 833 lira.

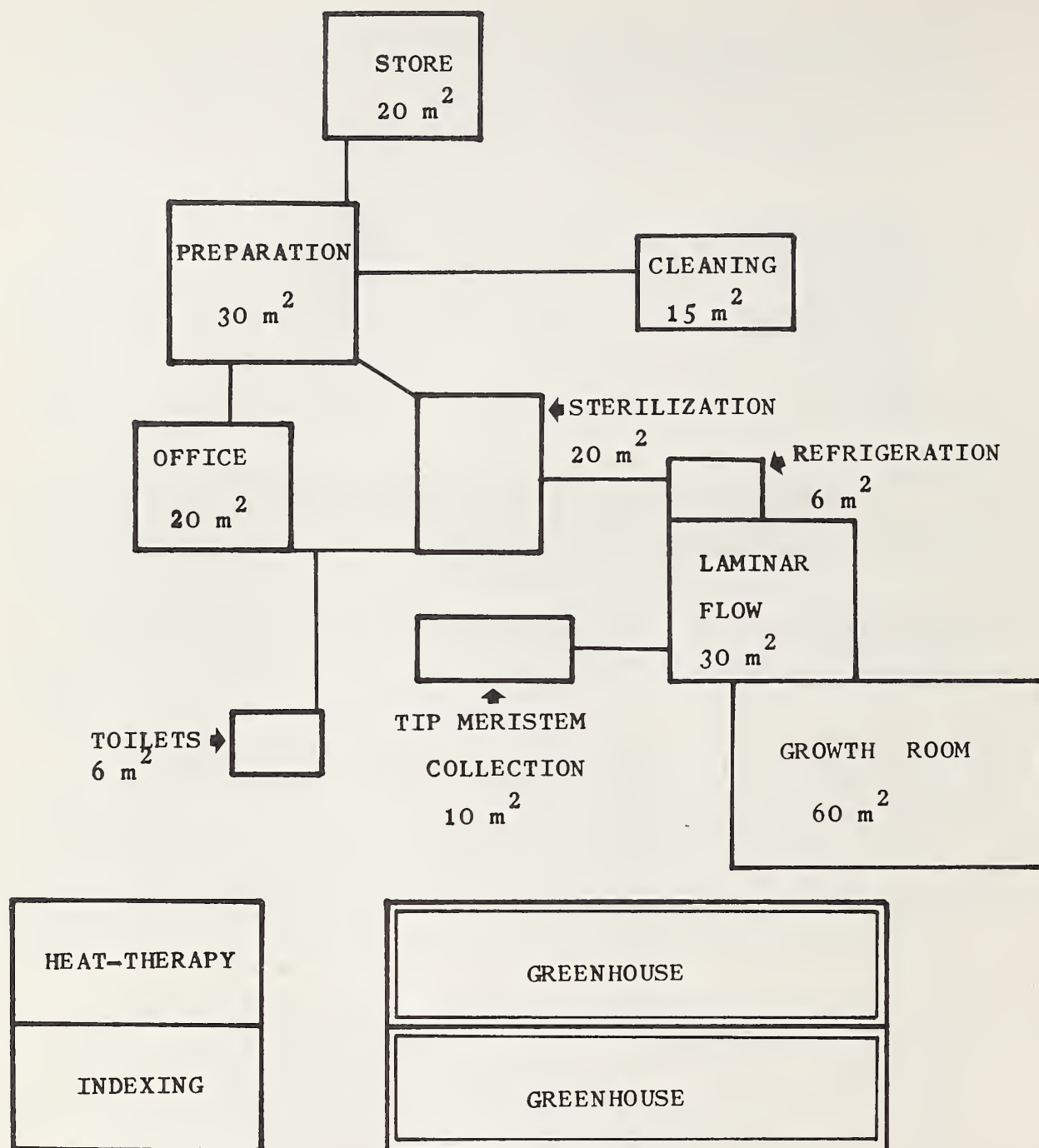


Figure 1. Design and space requirements for an idealized commercial tissue culture laboratory. (Note: 1 m<sup>2</sup> = 10.8 ft<sup>2</sup>)



Figure 2. Equipment used for collection of meristem tips.

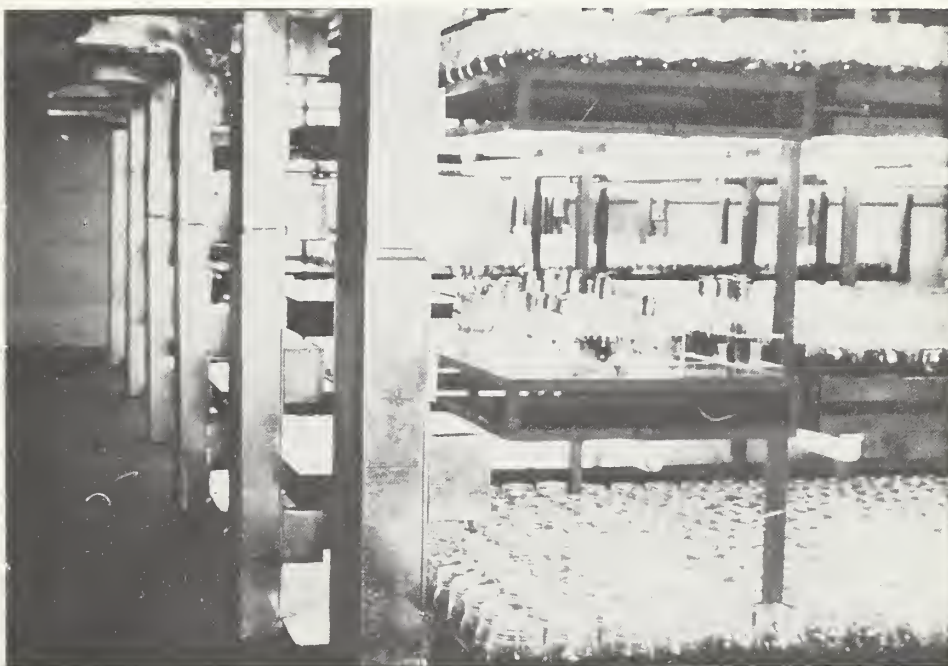


Figure 3. View of growth room in commercial tissue culture laboratory. Note provision for circulating cool air between fluorescent tubes and cultures on shelf above the lamps.

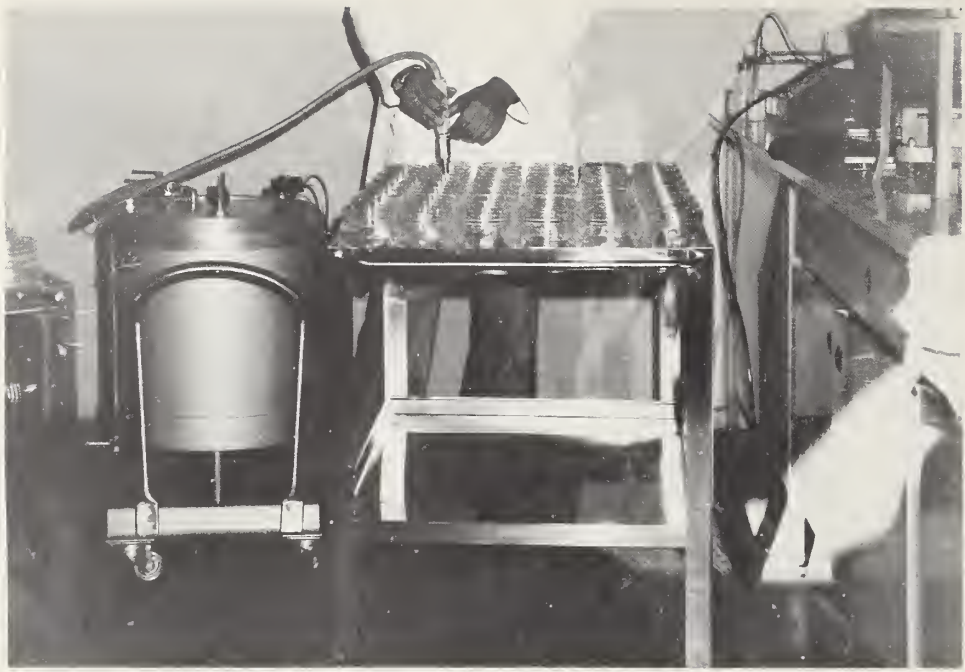


Figure 4. Apparatus for mixing and dispensing up to 25 liters of medium.



Figure 5. Micropropagated strawberry plantlets ready for acclimatization.



Figure 6. Ground beds in fiberglass greenhouse covered with plastic film for acclimatization of micropropagated strawberries.



Figure 7. Well-established strawberry plants after the plastic film has been removed.

# Potential Changes in Fruit Growing Resulting From Use of Tissue Culture

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With the exception of propagation, all production techniques presently used in orchards were developed during the last two decades. In contrast, propagation by grafting is at least 2,000 years old. Apostle Paul spoke about branches that had been grafted onto the olive tree (4). Pliny described cleft grafts as practiced in the first century by the Romans (6). From the 16th and 17th centuries, we have illustrations of grafting that are very similar to those we would make today (3). Now we are witnessing rapid changes even in propagation, as previous speakers have outlined during this conference.

As with every new technique in a complex production system, propagation by tissue culture also will bring with it a corresponding set of changes. We can anticipate the time when tissue culture propagation will give a new look to the fruit industry. Some of these changes we can foresee, since the seeds of the changes are already around us.

The changes will come soon in the nursery industry. Tissue culture propagation can speed the production of rootstocks. It can make M27 apple, Pixy plum, and Colt cherry rootstock available within a short period of time in quantities unheard of with conventional methods. We already have evidence that tissue culture material grows faster, and nurseries may save as much as a full year in production of a grafted tree. Grafting could become a year-round operation, easing the annual burden for everyone in the nursery.

In recent years, interest has increased in high- and ultrahigh-density orchards. Trees in such orchards are planted closely, are pruned by machines, and are short-lived compared to those in conventional plantings. Tree requirements for such orchards are high; occasionally as many as 4,000 trees per ha are needed. With present propagation practices, the cost of trees for high density orchards exceeds the value of the land. Thus, the cost of the trees is an obvious limiting factor in planting high density orchards. This cost is increased even more by the fact that high density orchards may last only for a relatively short time, often no more than 10 years.

Tissue culture has the potential to produce high quality trees relatively inexpensively. It is possible that growers may purchase small treelets (5-10" high), grow them for a year in their own nursery, and plant them in their permanent place mechanically. Tree-fruit nurseries thus would become a laboratory-greenhouse operation, for most of their

business will be selling small plants equivalent to tomato seedlings or rooted carnations, much the same way rootstock nurseries sell liners today.

Trees will stand on their own roots, an astonishing thought for many present-day horticulturists. We have enough information, at least in peaches, that growing own-rooted trees is not as impossible as once was thought. In Australia, 'Golden Queen' peach and 20 other cultivars performed as well on their own roots as did trees grafted on 'Elberta' seedlings. During the past 4-5 years, at least a half-million peach trees have been propagated on their own roots in Australia because of the interest in the Tatura trellis system. In Israel, at the Volcani Institute, experience has shown that peaches can be grown on their own roots. Slow-growing cultivars appear to be semidwarf on their own root, whereas they become large trees when grafted onto rootstocks (5). In Georgia, 8-year-old trees yielded equally, whether own-rooted or grafted. In Washington State, 5 own-rooted cultivars of peaches grew as well as grafted trees in an experimental planting; and in South Carolina, self-rooted trees of 'Loring' performed well even though the orchard was somewhat neglected (5). Pears soon may follow the peach. 'Bartlett', the most important cultivar, is normally budded on 'Bartlett' seedlings. Thus, the need for grafting is not obvious.

Rootstocks are important in three aspects. First, rootstocks can reduce tree size. While this aspect is largely unknown for all tree fruits other than apple and pear, it is important for these crops. In parts of Western Europe semi-dwarf and dwarf stocks are employed to contain tree size, whereas in North America, Eastern Europe, and the entire southern hemisphere the semi-standard and even standard stocks continue to predominate. With pears, the use of quince for dwarfing is limited to favorable soils. A large number of apple cultivars, e.g., 'Golden Delicious', 'Jonathan', 'Idared', and many others, are likely to be semi-dwarf on their own roots, thus producing the tree size that is most popular today. Second, rootstocks can also provide resistance to soil diseases, insects which damage roots, and nematodes, each of which can have importance in certain locations. However, these problems do not have overriding overall importance. Nemaguard, a nematode-resistant peach rootstock, has been virtually eliminated from use in the nematode-infested soils of the Southeast in favor of Lovell and Halford, two nematode-sensitive rootstocks, because Nemaguard sensitizes the scion to cold injury resulting in damage which has become intolerable. Third, rootstocks can also compensate for adverse soil conditions, e.g., high soil pH and high water table. In Italy, almond-peach hybrids are better rootstocks than peach because the hybrids resist chlorosis better than peach seedlings on high pH soils. However, some cultivars grafted on peach show less chlorosis than others. The possibility exists that those peach cultivars which do not suffer from chlorosis on alkaline soils when grafted on peach would also be satisfactory on their own roots. A similar analogy can be used for pear cultivars which become chlorotic if grafted on common pear rootstock.

Elimination of the graft union may increase the translocation within the tree. Even the most compatible unions appear to be a deterrent to Ca uptake. We have always found more Ca in leaves of the rootstock below the graft union than in the leaves on the scion. We have examined enough combinations to conclude that in addition to the rootstock and scion effects there is an unquestionable effect due to the graft union. Own-rooted trees, of course, would not have this disadvantage.

Grafting will remain a propagation technique reserved for special conditions where the characteristics of the rootstock are absolutely essential.

Because trees will be less expensive and therefore expendable, meaning that they can be discarded after a shorter lifetime, mechanical handling of all operations will increase. Crowding of branches caused by mechanical pruning will be a lesser problem because the orchards can be replaced after 6-10 years of production. This will increase the use of machinery and the number of orchards especially designed for mechanization.

Tissue culture propagated trees will be free from known latent viruses. In addition, they are generated from forced buds and they are likely to be vegetative in the early years of their life. Both of these characteristics tend to produce larger trees and extend the time between planting and production. Scientists must learn how to promote early production of own-rooted trees. Early production and overall productivity are related phenomena. It is very likely that by the time we are able to shorten the vegetative period we will also correspondingly increase productivity and attain yields never before seen.

Breeders will have a new tool with tissue culture. Mutation breeding using chemical mutagens incorporated in the media should be possible. Since hormonal regulation systems in plants (e.g., short-internode dwarfness) is one of the easily inducible mutations, we can look forward to the development of genetically dwarf cultivars. Tissue culture will also allow protoplast fusion, by which we can bridge interspecific and intergeneric gaps not possible before.

New cultivars will be introduced faster regardless of whether they are developed by conventional breeding techniques, new breeding methods, or introductions from abroad. The origination and testing of a new tree fruit or nut cultivar is a lifetime project; time needed from seed to introduction of small fruits such as blueberry is shorter, but is still estimated around 15-18 years; even the development of new strawberry cultivars requires about 10 years. Each of these intervals can be shortened by 30-50% if tissue culture propagation is used. It takes an equally long time to introduce imported cultivars commercially. With tissue culture propagation, any number of plants of a given cultivar can be produced within one year after the release of the cultivar from quarantine. Our new everbearing strawberries, EB 60 and EB 62, are being increased by tissue culture for distribution to nurseries. In six months

during the winter five or six thousand plants can easily be produced which can be multiplied 1 to 20 in the field giving 100,000 plants within one year. With a conventional system, it would have taken at least three years.

It takes a long time from the beginning of a new technique until its effects are fully developed. A time span of 20-25 years can be predicted before tissue culture propagation changes fruit production. The view we present here is visionary but the beginning has already occurred at many places, in many directions. We merely need to put together the details of the new systems. Scientists will toil for a long time before the forthcoming changes are realized. We can look forward to closely planted orchards, with genetically dwarfed trees on their own roots, in a highly mechanized system. Planting, pruning, and harvesting will all be done by machines and the orchards will be kept for a relatively short time, perhaps less than 10 years. The geographical areas of production may also change; unfavorable soils will be eliminated from production to avoid the need for special rootstocks. New ecotypes of the same cultivars will be developed more quickly and more easily than by conventional breeding, giving the growers a wider choice of well-adapted material. All these changes will be possible because of the breakthroughs in propagation of fruit producing plants outlined in this meeting.

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Table 1. Predicted effects on tissue culture propagation of fruits on production techniques.

Possible Advantages

Virus free plants will increase productivity.

Absence of graft union will increase ion uptake (Ca) enhancing quality of fruit.

Orchards will be high density with increased mechanization.

New cultivars, especially genetic dwarfs, will be developed, perhaps by mutation breeding, and such cultivars will be introduced rapidly.

Possible Disadvantages

Plants will be vegetative, and research is needed to force them to produce early which in turn could increase productivity.

Trees will vary in size according to the cultivar.

Roots of certain cultivars may be sensitive to pests and environmental conditions.

Geographic location of production will change to avoid unfavorable soils for which specific rootstocks would be needed.

Orchards will be of short duration - 6 to 10 years.

Mutations may occur in some cultivars such as 'Delicious' apple which is known to produce mutations. The extent to which mutation occurs or is permissible must be evaluated carefully.

Registrants - Tissue Culture Conference

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